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THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Michael Wayne Graham et al.

Serial No. : 10/821,726 Examiner : Vivlemore, Tracey A.

Filed : April 8, 2004 Art Unit : 1635

For : SYNTHETIC GENES AND GENETIC CONSTRUCTS

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New York, New York 10112

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Commissioner for Patents
P.O. Box 1450
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Sir:

DECLARATION OF DR. ARTHUR RIGGS

I, Dr. Arthur D. Riggs, declare as follows:

1. I am a Professor of Biology, Chair of the Department of Diabetes and Metabolic Diseases Research and Director Emeritus at the Beckman Research Institute of City of Hope, in Duarte, California. A copy of my *curriculum vitae* and a list of my publications are attached hereto as **Exhibit A**.

2. I have been retained by the Assignee's counsel as a technical expert in connection with the above-identified application. I am being compensated at $600.00 per hour (or a maximum of $5,000 for a day). I understand that a licensee of the subject application has a research agreement with certain investigators at the City of Hope. However, I am not otherwise affiliated with the Assignee or the licensee.

I. Scope of Opinion

3. I have been provided with, and asked to review, the Australian Provisional Patent Application No. PP2492 ("Applicants' Provisional"), the claims of U.S. Application 10/821,726 amended as indicated in **Exhibit B** hereto, the Office Action dated November 3, 2008, and the references cited within that Office Action, including the Fire et al. Provisional Patent Application No. 60/068,562 ("Fire Provisional"). I have also been provided with, and asked to review, PCT International Publication No. WO 94/01550, published January 20, 1994 ("Agrawal et al."), U.S. Patent No. 5,270,163, issued December 14, 1993 ("Gold et al."), U.S. Patent No. 5,580,703, issued December 3, 1996 ("Kotin et al."), and U.S. Patent No. 5,474,935, issued December 12, 1995 ("Chatterjee et al.").

4. I have been asked to provide my opinions of what would have been the views of a person of ordinary skill in the art as of March 1998. I believe I can accurately describe the perspective of such a person. In March 1998, I was actively involved in the areas of gene regulation and epigenetic mechanisms. For the purpose of this declaration I have understood that one skilled in this art in 1997 or 1998 would have at least a doctoral degree, e.g. a Ph.D. degree, in molecular biology or a related discipline, have post-doctoral training, have knowledge in cell biology, biochemistry, developmental biology and genetics, and be well trained in laboratory methodologies.

5. The opinions set forth in this declaration are based on my professional knowledge and expertise, as indicated in my *curriculum vitae*, my review of the Applicants' Provisional Application, and the Office Action dated November 3, 2008, including the documents cited in the Office Action, as well as additional documents cited in this declaration.

II. Disclosure of the Cited Prior Art

6. The Fire et al. Provisional is based on and incorporates the results published in the 1998 publication in Nature ("Fire et al. Nature publication") where RNA interference (RNAi) was first described in *C. elegans* (Fire et al, Nature, 1998, attached hereto as **Exhibit C**). When I first heard the results, I, along with many of my colleagues, was astonished that the injection of double-stranded RNA had such an effect on gene expression. In that publication, Fire et al. described the exogenous delivery of double-stranded RNA by injecting the RNA into the body cavity or gonads of *C. elegans*. Thus, the RNA was added extracellularly. Yet, as Fire et al. noted, "dsRNA-mediated interference showed a surprising ability to cross cellular boundaries" in *C.elegans*. Fire et al. also mention that injection into the cytoplasm of intestinal cells is effective, but no data supporting this methodology was reported. The double-stranded RNA was produced *in vitro* as separate strands, subsequently annealed, and was sequence specific for the target genes, covering lengths of 299 to 1033 nucleotides of the target genes. Injection of these molecules resulted in a silencing effect of the target gene expression that was one-hundred times more efficient than that observed by injecting antisense RNA alone, as evidenced by the fact that Fire et al. witnessed inhibition of gene expression with as little as "a few molecules [of double-stranded RNA] per cell." The observed gene silencing correlated with a decrease or elimination of the target mRNA, yet the mechanism by which the double-stranded RNA was causing this effect was unknown. Based on his observations, Fire et al. did indicate that "[a] simple antisense model is not likely: annealing between a few injected RNA molecules and excess endogenous transcripts would not be expected to yield observable phenotypes" (Fire et al., Nature, 1998). In the Fire et al. Provisional, Fire et al. confirm that their "invention differs from antisense-mediated interference in both approach and effectiveness."

7. Reviewing the disclosure of the Fire et al. Provisional Application, it was evident to me that Fire et al. included several generalized extensions of his RNA interference discovery, most likely due to a lack of mechanism to describe the results that he had observed. For example, although Fire et al. Provisional Application mentions mammalian cells, the experimental data reported would not have motivated one skilled in the art to add 299 to 1033 base-pair double-stranded RNA to the cytoplasm of mammalian cells because it was known in that art that these cells mounted a non-specific response to double-stranded RNA that would globally inhibit gene expression. The Fire et al. Provisional purports that all of the readily conceivable molecular biology modifications would be tolerated by the experimental system of Fire et al. If one of ordinary skill in the art considered all of the choices offered by the Fire et al. Provisional, a very large number of permutations are apparent. Confounding an attempt to select any permutation is the lack of a clear preference or suggestion, other than in the examples, for one combination of elements over another. While numerous combinations are disclosed as possible, there is no disclosure of which combinations other than the exemplified combinations, would work. Thus, aside from knowing that the technique as exemplified would work in *C. elegans*, one of skill in the art would be no closer to successfully modifying the technique after reading the Fire et al. Provisional than they would have been if they simply read the Fire et al. Nature publication. Moreover, if one looked to the references in the art at the time, they would also fail to find relevant guidance because practically nothing else was known about the then very new observations reported by Fire et al.

8. One might have come across antisense RNA technology, which was another approach to gene silencing that was technically different from interference described by Fire et al. The Examiner has cited Agrawal et al. and Chatterjee et al. from the antisense art to argue that the Applicants' invention was obvious at the time. However, it was clear that the

interference described by Fire et al. was not working via an antisense mechanism, thus invalidating the theoretical parallel drawn by the Examiner. Antisense technology required the hybridization of the antisense molecule with its target DNA or mRNA and was highly dependent on the concentration of antisense molecules in the cells. Agrawal et al. describe the design and use of small chemically synthesized antisense oligonucleotides and their effectiveness for inhibiting gene expression. Chatterjee et al. describe the use of vectors derived from Adeno-associated virus for the delivery of antisense oligonucleotides to a target cell. In my opinion, these references do not offer any teaching that one of ordinary skill in the art at the time could have applied to predictably modify the teachings of Fire et al. to produce the Applicants' invention.

9. It is my opinion that one of skill in the art at the time, looking to practice RNA-mediated gene silencing, would not have sought out or relied on the teachings of Gold et al. or Kotin et al. Gold et al. discuss a method to identify nucleic acid ligands of proteins. They produce RNA stem loops that have a protein binding site in their loop region. Gold et al. do not suggest using these molecules for RNA interference. Kotin et al. describe the cloning of a piece of a viral genome, which could be used as a probe to detect integration of the virus in a host genome. Again, Kotin et al. do not discuss or suggest the use of their invention to achieve RNA interference. In my opinion, these references do not offer any teaching that one of ordinary skill in the art at the time could have applied to modify the teachings of Fire et al. to predictably produce the Applicants' invention.

III. Interpretation of the Claims

10. My interpretation of the claims, attached hereto as **Exhibit B,** is based on my understanding of how one of ordinary skill in the art would have understood the terms appearing

in the claims in the context of the claims as a whole, in view of the description of the invention set forth in the patent.

11. In general terms, the claims relate to a process for using double-stranded DNA constructs to reduce target gene expression in mammalian cells. More specifically, claim 134 describes in detail a process for delaying, repressing or reducing the expression of a target gene in a mammalian cell using a double-stranded DNA construct. The double-stranded DNA construct has a single promoter that is operable in mammalian cells, which drives the transcription of a single DNA sequence that has three sub-sequences: (1) a sequence ("structural gene sequence") that has 20-30 consecutive nucleotides that are identical to the sequence of a target gene; (2) an intervening sequence or "stuffer fragment" distinct from either sub-sequence (1) or (3); and (3) a sequence that is the inverted repeat of the first sub-sequence ("20-30 consecutive nucleotides identical in sequence to, and in an inverted orientation relative to, the 20-30 nucleotides of the first structural gene sequence"). It is my understanding that the transcription of this construct would, in a cell, result in a single RNA strand that forms a hairpin structure within which sub-sequence corresponding to (1) and sub-sequence corresponding to (3) are in a duplexed state and the distinct stuffer (sub-sequence (2)) forms a loop at one end of the hairpin. It is also my understanding that the loop formed by the stuffer allowed for complete intramolecular base-paring between sub-sequence (1) and sub-sequence (3). Following transfection of the DNA construct into mammalian cells, the DNA construct will be transcribed in the nucleus and the produced RNA hairpin would be capable of repressing or reducing expression of the target gene.

12. A number of dependent claims further specify the details of the process and the double-stranded DNA construct used in the process described in claim 134. Claims 135 to 143

further specify types of possible target genes for the process of claim 134, including viral genes, transgenes or endogenous genes. Claims 144 and 145 further specify what regions of a target gene can be targeted in the process of claim 134. Claim 146 further specifies that the structural gene sequences of the construct of the process of claim 134 can be arranged as an interrupted palindrome. Claims 147 to 149 further specify the length of the nucleotides that separate the structural gene sequences within the palindrome of claim 146. Claims 150 to 152 further specify methods to introduce the double-stranded DNA construct into mammalian cells. Claim 153 further specifies that the process of claim 134 takes place in a mammalian cell. Claim 154 further specifies that the DNA construct of the process of claim 134 is integrated into the genome of the targeted mammalian cell.

IV. Obviousness Rejections

13. I have read the Examiner's arguments in the November 3, 2008 Office Action offered in support of the obviousness rejection of the Applicants' invention over Fire et al. Patent in view of Agrawal et al., Chatterjee et al., Gold et al., and Kotin et al., and I respectfully disagree with the Examiner's conclusions. While it is clear that Fire et al. described the use of double-stranded RNA to inhibit gene expression, the methods by which they did so significantly differ from those claimed in the current invention and provide no evidence that would predict the success of the Applicants' invention in March 1998. Additionally, the combination of the secondary references cited by the Examiner would not have aided one of skill in the art in developing a successful DNA construct for RNA interference.

14. At the time of the Fire et al. Nature publication, those skilled in the art were mystified by what Fire et al. had described. Since such an experimental system had never been reported before, the only knowledge in the art was derived from the Fire et al. Nature

publication. Therefore, it was impossible to predict how the results would be affected by changes made to the experimental system reported by Fire et al. At that time, numerous issues concerning perturbations to Fire's system would be readily apparent and be a cause for concern to one of ordinary skill in the art, including:

(1) How would changes in the delivery technique of the RNA affect the results?

(2) How would changes to the RNA secondary structure affect the results?

(3) How would changes to the size of the double stranded RNA affect the results?

(4) How would endogenous production of double-stranded RNA affect mammalian cells?

As one considers the above questions, numerous variables become apparent that could impact the ability of a RNA molecule to promote the effects reported by Fire et al. These same variables would have prevented one from predicting the effects of the proposed changes. I describe a number of these variables below.

Changes in the delivery technique of the RNA

15. Fire et al. delivered RNA that had been synthesized *in vitro* to target cells via injection. It was known in the art that a certain level of delivery efficiency was associated with injecting nucleic acid molecules into cells or into the body cavity of *C. elegans*. It was also known that the efficiency of introducing nucleic acid molecules varied amongst different introduction techniques, such as electroporation, lipid-mediated carrier transport or chemical-mediated transport. It was unknown at the time whether the effects observed by Fire et al. would have been obtained if one used one of these other methods of introducing double-stranded RNA into the cells.

16. As an extension of the delivery question, one may have asked if the double-stranded RNA could have been produced in the cell and, if so, how. Intracellular production could happen in the cytoplasm, via various viral vectors, or it could happen in the nucleus, where most RNA production occurs. Producing RNA in the nucleus presents a number of additional unknowns that would have to be resolved to recapitulate the original scenario demonstrated by Fire et al., *i.e.* double-stranded RNA introduced to the cytoplasm. The following are examples of such unknowns:

17. *The double-stranded RNA may get trapped in the nucleus.* It was known to those skilled in the art that multiple proteins interacted with single-stranded mRNA in the nucleus to mediate its translocation through the nuclear pore to the cytoplasm. It was possible that the canonical export machinery would not have recognized or bound to double-stranded RNA to facilitate its transport out of the nucleus. Alternatively, there may have been nuclear retention factors that could bind the duplex RNA to prevent it from leaving the nucleus. For example, Okano et al. reported that antisense RNA specific for the myelin basic protein formed duplexes in the nucleus and suggested that duplex formation inhibited transport of mRNA from the nucleus (Okano H. et al., J. Neurochem., 1991, attached hereto as **Exhibit D**). It was possible that double-stranded RNA produced in the nucleus could suffer the same fate.

18. *Double-stranded RNA produced in the nucleus may get modified.* Double-stranded RNA produced in the nucleus would be exposed to various nucleus-specific enzymes that could modify the RNA. One example of such an enzyme is a nuclear double-stranded RNA dependent adenosine deaminase. In the nucleus, this enzyme targets the double-stranded RNA portions of duplexes and converts adenosine (A) to inosine (I), which makes the duplex unstable. The instability of the duplex may lead to its unwinding, which would expose the unwound strands to

further causes of degradation (Kumar M and Carmichael G, Microbiol. Mol. Biol. Rev., 1998, attached hereto as **Exhibit E**). Thus, unwinding of the duplex would have destroyed the important double-stranded structure of the RNA, and would be thought to render the RNA incapable of mediating gene silencing. Additionally, the incorporation of inosine in the RNA would have decreased the stringency of the intramolecular base-pairing within the duplex, which could have resulted in a heterogeneous collection of imperfect duplexes and other aberrant RNA secondary structures in the nucleus. Because one of ordinary skill in the art could not predict the effect that inosines would have on the system reported by Fire et al., it would have been difficult to predict if inosine-containing RNA duplexes produced in the nucleus would function.

19. *Double-stranded RNA produced in the nucleus may get degraded.* At the time of the present invention, a major concern with introducing RNA into cells was the degradation of that RNA. Most of RNA degradation machinery in the cytoplasm was suspected to target single stranded RNA at the 5' or the 3' termini. Double stranded RNA in the cytoplasm was generally thought to be resistant to this type of degradation. However, production of double-stranded RNA in the nucleus exposed that RNA to specialized RNAses that targeted double-stranded RNA, such as RNAse III (Wu H et al., J. Biol. Chem., 1998, attached hereto as **Exhibit F**). These enzymes would specifically lead to the degradation of duplex RNA in the nucleus, but not of the duplex RNA directly introduced into the cytoplasm by Fire et al. It could not have been predicted whether duplex structures would be degraded in the nucleus, thus compromising the double-stranded RNA and the ability to specifically silence genes as reported by Fire et al.

20. *Polyadenylation at the 3' end of RNA may interfere with RNA interference.* Messenger RNA precursors (pre-mRNA) that are produced in the nucleus are modified at their 3'terminus by the addition of a polyadenylation signal (poly-A tail) of ~200-250 adenine

residues (Lodish et al., Molecular Cell Biology, c1999, attached hereto as **Exhibit G**). At the time of the present invention, two proposed functions of the poly-A tail were: (1) to protect the transcript from degradation (Sachs A. and Wahle E. J. Biol. Chem. 1993, attached hereto as **Exhibit H**); and (2) to stimulate transportation out of the nucleus (Huang Y. and Carmichael G., Mol. Cell. Biol., 1996, attached hereto as **Exhibit I**). Therefore, it would have been presumed to be important for RNA produced in the nucleus to have a poly-A tail for protection and transport out of the nucleus. However, one skilled in the art would not have been able to predict the effect that a poly-A tail would have had on the ability of an RNA duplex to mediate the interference reported by Fire et al. The poly-A tail would lead to a large, single-stranded overhang on both strands of the RNA duplex produced in the nucleus. Because the RNA duplexes of Fire et al. did not have a poly-A tail, it was not possible to predict how this structure would affect the function of the RNA duplex.

21. *Heterogeneous nuclear ribonucleoproteins may affect double-stranded RNA formation.* When pre-mRNA is produced in the nucleus, it is quickly bound by numerous heterogeneous nuclear ribonucleoproteins (hnRNPs). One function of these proteins is to promote the correct processing of endogenous pre-mRNA by preventing folding of the RNA onto itself and the formation of secondary structures. (Lodish et al., Molecular Cell Biology, c1999, attached hereto as **Exhibit G**). A linearized RNA molecule is more easily accessible to the factors responsible for 5' end capping, splicing, and 3' end polyadenylation. Thus, as RNA of the present invention was transcribed in the nucleus, hnRNPs would be thought to bind to the RNA and prevent it from base-pairing with its complementary strand. If the RNA was maintained in a linear form, it may not have been able to promote the effects reported by Fire et al. with RNA that was introduced in a double-stranded form. Additionally, the linear RNA

would have been more susceptible to single strand specific RNAses in the nucleus as well as in cytoplasm.

22. *Binding of heterogeneous nuclear ribonucleoproteins may inhibit RNA interference function.* Additionally, it was known that some hnRNPs from the nucleus remain associated with mRNA as it is translocated into the cytoplasm (Lodish et al., Molecular Cell Biology, c1999, attached hereto as **Exhibit G**). Since the mechanism of the interference reported by Fire et al. was unknown, it was not possible to predict at the time if proteins bound to the RNA would have an effect on its ability to cause gene silencing. Possible consequences of bound nuclear proteins are: (1) they could obstruct a cleavage site that might have been important; (2) they could obstruct a binding site or interaction domain for other proteins that played a role; (3) they could physically block other proteins from interacting with the RNA; or (4) they could interfere with a potential interaction between the RNA and its target. Thus, if the RNA was able to assume a duplex structure, but the nuclear proteins remained bound to the RNA duplex when it encountered the silencing targets or unknown effectors in the cytoplasm, the bound duplex may not have been able to function properly to cause silencing.

Changes to the RNA secondary structure

23. Fire et al. explained that the double-stranded structure of the RNA was critical to the reported effects; therefore, one of ordinary skill in the art could not predict how the manipulation of that structure would influence gene silencing. Possible alternative RNA structures with double-stranded features include hairpins/stem loops, pseudoknots, hammerheads and cloverleafs, each of which could be formed by a single strand of RNA. There is no information in the Fire et al. Provisional or in the Fire et al. Nature publication which would allow one of ordinary skill to predict whether any of the other structures, and most importantly

whether two separate complementary RNA strands, were required for activity. If two separate strands of RNA were needed for the RNA interference mechanism, for example, if the unknown mechanism required that the two strands separate *in vivo*, then one of ordinary skill would question whether a single, self-complementary strand would function. Alternatively, if two free ends of the RNA molecule were required for the unknown mechanism of the interference reported by Fire et al., then one would question whether a single self-complementary RNA strand would function. Thus, it could not be predicted at the time whether the hairpin RNA structure produced by the DNA constructs of the present invention would be able to cause the interference effect reported by Fire et al.

Changes to the size of the double stranded RNA

24. The shortest RNA with which Fire et al. observed interference was 299 base pairs long. Although the Fire Provisional discloses a range of RNA lengths from 25 to 400 base pairs corresponding to the target gene, Fire et al. neither suggest, nor demonstrate if any of the lengths encompassed by that range is optimal. Furthermore, it was totally unknown whether double-stranded RNA molecules shorter than 299 nucleotides could operate in RNA interference, as no results for such experiments had been reported at the time. The present invention teaches the use of DNA constructs that would produce a hairpin RNA having a duplex region 20-30 base pairs long. There was no indication that the use of RNAs an order of magnitude shorter than those shown to work by Fire et al. would have caused the same result. If a certain length of nucleotides within the double-stranded RNA was required for RNA interference, then small double-stranded RNA molecules below the minimum size requirement would not operate by the unknown mechanisms of the system. Because the mechanism was not known, the size

requirements were a mystery and one could not predict the consequence of decreasing the length of the RNA duplex.

Change to expressing double-stranded RNA in mammalian cells

25. At the time of Applicants' invention, there was widespread uncertainty in the art about whether double-stranded RNA could be delivered to mammalian cells without causing the cells to die. It was known in the art that double-stranded RNA caused the interferon response in mammalian cells which led to global mRNA degradation and translation inhibition (Reviewed in Jacobs B.L. and Langland J.O., Virology, 1996, attached hereto as **Exhibit J**). Because this response existed, it was impossible to predict whether double-stranded RNA would cause specific RNA interference in mammalian cells without triggering the non-specific double-stranded RNA response. Thus, any attempts to induce specific RNA interference in mammalian cells could not have been viewed as obvious due to the prevailing belief in the art at the time that RNA interference would not be possible if the double-stranded RNA response was functioning in the targeted mammalian cells.

26. The uncertainties imposed by deviating from the teachings of Fire et al. were significant. Consequently, it was not possible for one of ordinary skill in the art prior to Applicants' invention to predict if the invention claimed by Applicants would have succeeded in causing any interference effect. The Applicants' Provisional was the first to disclose that the specific combination of elements as claimed would cause interference. The Examiner's reliance on Agrawal et al, Chatterjee et al, Gold et al. and Kotin et al., which come from irrelevant arts, would not have alleviated the concerns of those of ordinary skill in the art which I have described above.

27. In conclusion, I believe that there is no information in the Fire et al. Provisional, the Fire et al. Patent, Agrawal et al., Chatterjee et al., Gold et al., or Kotin et al. which would have permitted one of ordinary skill in the art to reasonably expect prior to Applicants' invention that the specific combination of features of the Applicants' claimed invention would result in any interference. Specifically, Applicants' selection of a process using DNA constructs comprising structural gene regions with a targeting region of 20-30 nucleotides to produce double-stranded RNA in mammalian cells to cause RNA interference was not disclosed by Fire et al. and one of skill in the art at the time would not have expected such a combination to succeed, despite the disclosure of Fire et al. I firmly believe that the endogenous production of RNA as claimed for the purpose of inhibiting expression of the target gene was a novel and non-obvious invention at the time the Applicants filed their Provisional application, *i.e.* on March 20, 1998.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made herein on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-identified patent.

A Riggs

Arthur D. Riggs, Ph.D.

10/2/09

Date

EXHIBIT A

Declaration of Dr. Arthur Riggs

Submitted: October 7, 2009

Serial No. 10/821,726

Filed: April 8, 2004

Applicants: Michael Wayne Graham et al.

CURRICULUM VITAE

ARTHUR D. RIGGS, PH.D.

Born: August 8, 1939, Modesto, California.
A.B.: University of California at Riverside, 1961, Chemistry.
Ph.D.: California Institute of Technology, Pasadena, 1966, Biochemistry.

Professional Experience

1961-1966: Predoctoral Fellow, Biology Department, Calif. Inst. of Tech., Pasadena, CA.
1966-1969: Postdoctoral Fellow, Salk Institute for Biological Studies, La Jolla, CA.
1969-1974: Associate Research Scientist, Department of Molecular Biology, City of Hope National Medical Center, Duarte, CA.
1974-1983: Senior Research Scientist, Dept. of Molecular Biology, City of Hope Med. Ctr.
1978-1995: Adjunct Professor, University of Southern California, Los Angeles, Calif.
1979-1981: Associate Chair, Division of Biology, City of Hope Medical Center.
1981-1983: Chair, Division of Biology, City of Hope Medical Center
1983-1987: Chair, Division of Biology, Beckman Research Institute of the City of Hope
(In 1983 the City of Hope's research institute was renamed the Beckman Research Institute of the City of Hope.)
1994-2000: Chair, Division of Biology, Beckman Research Institute of the City of Hope
1981-1987: Associate Director for Laboratory Research, City of Hope Cancer Center.
1993-1995: Director, Shared Resources of City of Hope Cancer Ctr.
1994-1998: Founding Dean, City of Hope Graduate School
1998-1999: Associate Director for Research, City of Hope Medical Center
2000-2007: Director, Beckman Research Institute of the City of Hope
2008-present: Director Emeritus and Professor of Biology, Beckman Research Institute of the City of Hope
2008-present: Chair, Department of Diabetes and Metabolic Disease Research

Honors & Awards

High honors in chemistry, University of California, Riverside, 1960.
National Science Foundation, Predoctoral Fellow, 1961-1964.
National Institutes of Health Postdoctoral Fellow, 1966-1969.
Juvenile Diabetes Foundation Research Award, 1979.
Gallery of Achievement Award, City of Hope, 1981
Distinguished Alumni Award, Univ. Calif., Riverside, 1988
Technology Leadership Award, 2004
Member National Academy of Sciences, 2006
Distinguished Alumni Award, California Institute of Technology, 2008

Principal Research Interest:

Gene regulation and mammalian development, X-chromosome inactivation, DNA methylation, and epigenetic mechanisms.

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Patents

	PAT. NO.	Issued	Title
1	7,083,926	2006	Method for identifying accessible binding sites on RNA
2	7,033,763	2006	Pyrophosphorolysis activated polymerization (PAP)
3	6,562,570	2003	Method for identifying accessible binding sites on RNA
4	6,331,415	2001	Methods of producing immunoglobulins, vectors and transformed host cells for use therein
5	6,242,567	2001	Method for detection and prevention of human cytomegalovirus infection
6	6,133,433	2000	Method for detection and prevention of human cytomegalovirus infection
7	5,583,013	1996	Method and means for microbial polypeptide expression
8	5,420,020	2001	Method and means for microbial polypeptide expression
9	5,270,183	1993	Device and method for the automated cycling of solutions between two or more temperatures
10	5,221,619	1993	Method and means for microbial polypeptide expression
11	5,081,235	1992	Chimeric anti-CEA antibody
12	5,075,431	1991	Chimeric anti-CEA antibody
13	5,075,213	1991	Method for detection and prevention of human cytomegalovirus infection
14	4,816,567	1989	Recombinant immunoglobin preparations
15	4,812,554	1989	Somatostatin peptide conjugate
16	4,704,362	1987	Recombinant cloning vehicle microbial polypeptide expression
17	4,563,424	1986	Method and means for somatostatin protein conjugate expression
18	4,431,739	1984	Transformant bacterial culture capable of expressing heterologous protein
19	4,425,437	1984	Microbial polypeptide expression vehicle
20	4,366,246	1982	Method for microbial polypeptide expression

EXHIBIT B

Declaration of Dr. Arthur Riggs

Submitted: October 7, 2009

Serial No. 10/821,726

Filed: April 8, 2004

Applicants: Michael Wayne Graham et al.

October 4, 2009 Pending Claims

1-133. (Canceled)

134. (Currently amended) A process for ~~producing an RNA molecule which is capable of~~ delaying, repressing or otherwise reducing the expression of a target gene in a mammalian cell comprising introducing into a cell a double-stranded ~~synthetic gene~~ DNA construct consisting of a promoter operable in the cell, a transcription termination sequence active in the cell, and operably connected thereto

a first structural gene sequence comprising 20-30 consecutive nucleotides identical in sequence to a region of a target gene in the mammalian cell;

a second structural gene sequence comprising 20-30 consecutive nucleotides identical in sequence to, and in an inverted orientation relative to, the 20-30 consecutive nucleotides of the first structural gene sequence, thereby providing a repeating sequence ~~of~~ which is only 20-30 consecutive nucleotides in length; and

~~optionally~~ a stuffer fragment which~~, if present,~~ separates and links the first and second structural gene sequences,

~~wherein the a repeating sequence within the first and second structural gene sequences, and if present stuffer fragment, is only 20-30 nucleotides in length,~~

such that the ~~synthetic gene~~ double-stranded DNA construct is transcribed to produce the RNA molecule.

135. (Previously presented) The process of claim 134, wherein the region of the target gene is in an exon.

136. (Previously presented) The process of claim 134, wherein the target gene is a viral gene.

137. (Previously presented) The process of claim 136, wherein the viral gene encodes a DNA polymerase, RNA polymerase, or viral coat protein.

138. (Previously presented) The process of claim 134, wherein the target gene is from a lentivirus.

139. (Previously presented) The process of claim 134, wherein the target gene is from an immunodeficiency virus.

140. (Previously presented) The process of claim 134, wherein the target gene is from a single-stranded (+) RNA virus.

141. (Previously presented) The process of claim 134, wherein the target gene is from a double-stranded DNA virus.

142. (Previously presented) The process of claim 134, wherein the target gene is a transgene in the mammalian cell.

143. (Previously presented) The process of claim 134, wherein the target gene is an endogenous gene in the mammalian cell.

144. (Previously presented) The process of claim 134, wherein the 20-30 consecutive nucleotides are identical to a coding region of the target gene.

145. (Previously presented) The process of claim 134, wherein the 20-30 consecutive nucleotides are identical to a 5′- or 3′-untranslated sequence of the target gene.

146. (Currently Amended) The process of claim 134~~, wherein the stuffer fragment is present,~~ wherein the first structural gene sequence, the stuffer fragment and the second structural gene sequence form an interrupted palindrome sequence, and wherein the ~~repeated~~ repeating sequence of the interrupted palindrome sequence is only 20-30 consecutive nucleotides in length.

147. (Previously presented) The process of claim 146, wherein the stuffer fragment is a sequence of nucleotides 10-50 nucleotides in length.

148. (Previously presented) The process of claim 146, wherein the stuffer fragment is a sequence of nucleotides 50-100 nucleotides in length.

149. (Previously presented) The process of claim 146, wherein the stuffer fragment is a sequence of nucleotides 100-500 nucleotides in length.

150. (Currently Amended) The process of claim 134, wherein the double-stranded ~~synthetic gene~~ DNA construct is introduced by a virus particle.

151. (Currently Amended) The process of claim 134, wherein the double-stranded ~~synthetic gene~~ DNA construct is introduced by a liposome.

152. (Currently Amended) The process of claim 134, wherein the double-stranded ~~synthetic gene~~ DNA construct is introduced by transfection.

153. (Previously presented) The process of claim 134, wherein

the cell is the mammalian cell.

154. (Currently Amended) The process of claim 134, wherein the double-stranded ~~synthetic gene~~ DNA construct is integrated into the genome of the cell.

155. (New) A process for delaying, repressing or otherwise reducing the expression of a target gene in a mammalian cell comprising introducing into a cell a double-stranded DNA construct consisting of a promoter operable in the cell, a transcription termination sequence active in the cell, and operably connected thereto

a first structural gene sequence comprising 20-30 consecutive nucleotides identical in sequence to a region of a viral DNA polymerase gene, a viral RNA polymerase gene, a viral coat protein gene, or a visually-detectable gene involved in determining an external phenotype in the mammalian cell;

a second structural gene sequence comprising 20-30 consecutive nucleotides identical in sequence to, and in an inverted orientation relative to, the 20-30 consecutive nucleotides of the first structural gene sequence, thereby providing a repeating sequence which is only 20-30 consecutive nucleotides in length; and

a stuffer fragment which separates and links the first and second structural gene sequences,

wherein a repeating sequence within the double-stranded DNA construct is only 20-30 nucleotides in length,

such that the double-stranded DNA construct is transcribed to produce the RNA molecule.

EXHIBIT C

Declaration of Dr. Arthur Riggs

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and 10 $Na_3HP_2O_7$. FV solution also contained 0.2 NaF and 0.1 Na_3VO_4. Rarely, irreversible current rundown still occurred with FVPP. The total Na^+ concentration of all cytoplasmic solutions was adjusted to 30 mM with NaOH, and pH was adjusted to 7.0 with *N*-methylglucamine (NMG) or HCl. PIP_2 liposomes (20–200 nm) were prepared by sonicating 1 mM PIP_2 (Boehringer Mannheim) in distilled water. Reconstituted monoclonal PIP_2 antibody (Perspective Biosystems, Framingham, MA) was diluted 40-fold into experimental solution. Current–voltage relations of all currents reversed at E_K and showed characteristic rectification, mostly owing to the presence of Na^+ in FVPP and possibly also residual polyamines. Current records presented (measured at 30 °C, −30 mV holding potential) are digitized strip-chart recordings. Purified bovine brain $G\beta\gamma$[29] was diluted just before application such that the final detergent (CHAPS) concentration was 5 μM. Detergent-containing solution was washed away thoroughly before application of PIP_2, because application of phospholipid vesicles in the presence of detergent usually reversed the effects of $G\beta\gamma$; presumably, $G\beta\gamma$ can be extracted from membranes by detergent plus phospholipids.

Molecular biology. R188Q mutation was constructed by insertion of the mutant oligonucleotides between the *Bsm*I and *Bgl*II sites of pSPORT–ROMK1 (ref. 11). A polymerase chain reaction (PCR) fragment (amino acids 180–391) from pSPORT–ROMK1 R188Q mutant was subcloned into pGEX-2T vector (Pharmacia) for expression of R188Q mutant protein of GST–RKC. The construction, expression and purification of GST–IKC (amino acids 182–428 of IRK1), GST–GKC (180–462 of GIRK1), GST–IKN (1–86 of IRK1) have been described[21,22].

***In vitro* PIP_2 binding assay.** ^{3}H-PIP_2 in chloroform-methanol (1:1) (American Radiolabeled Chemicals; 0.4 μCi nM^{-1} specific activity) was dried under N_2 and sonicated in 100 μl phosphate buffered saline (PBS) to form pure ^{3}H-PIP_2 liposomes. Purified GST fusion protein (100 nM) was incubated with ^{3}H-PIP_2 (0.2–1 μM) and precipitated by glutathione 4B-Sepharose beads. After 1 wash with PBS, the precipitates were dissolved in SDS gel loading buffer and counted in a beta-scintillation counter using a window for ^{3}H. The bound ^{3}H radioactivity was typically in the range ~2–8% of the total added. For co-immunoprecipitation, 25% PIP_2 or PIP in 75% phosphatidylcholine (PC) background (30 μg PIP_2 or PIP (Boehringer Mannheim) and 90 μg phosphatidylcholine (Sigma)), both in chloroform, were dried down together and sonicated in 300 μl PBS to form mixed liposome. GST fusion proteins were first incubated with 25% PIP_2 or PIP liposome (100 μM) and PIP_2 antibodies (1:100 dilution) for 2 h and with protein A–Sepharose for a further 30 min. After one wash with PBS, the immunoprecipitates were separated by 10% SDS–PAGE, probed with specific antibodies[21,22], and visualized by ECL (Amersham). Each experiment was performed at least twice with similar results. The relative amount of immunoreactivity in each lane was quantified by serial dilutions of sample[21].

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Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*

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Experimental introduction of RNA into cells can be used in certain biological systems to interfere with the function of an endogenous gene[1,2]. Such effects have been proposed to result from a simple antisense mechanism that depends on hybridization between the injected RNA and endogenous messenger RNA transcripts. RNA interference has been used in the nematode *Caenorhabditis elegans* to manipulate gene expression[3,4]. Here we investigate the requirements for structure and delivery of the interfering RNA. To our surprise, we found that double-stranded RNA was substantially more effective at producing interference than was either strand individually. After injection into adult animals, purified single strands had at most a modest effect, whereas double-stranded mixtures caused potent and specific interference. The effects of this interference were evident in both the injected animals and their progeny. Only a few molecules of injected double-stranded RNA were required per affected cell, arguing against stochiometric interference with endogenous

mRNA and suggesting that there could be a catalytic or amplification component in the interference process.

Despite the usefulness of RNA interference in *C. elegans*, two features of the process have been difficult to explain. First, sense and antisense RNA preparations are each sufficient to cause interference[3,4]. Second, interference effects can persist well into the next generation, even though many endogenous RNA transcripts are rapidly degraded in the early embryo[5]. These results indicate a fundamental difference in behaviour between native RNAs (for example, mRNAs) and the molecules responsible for interference. We sought to test the possibility that this contrast reflects an underlying difference in RNA structure. RNA populations to be injected are generally prepared using bacteriophage RNA polymerases[6]. These polymerases, although highly specific, produce some random or ectopic transcripts. DNA transgene arrays also produce a fraction of aberrant RNA products[3]. From these facts, we surmised that the interfering RNA populations might include some molecules with double-stranded character. To test whether double-stranded character might contribute to interference, we further purified single-stranded RNAs and compared interference activities of individual strands with the activity of a deliberately prepared double-stranded hybrid.

The *unc-22* gene was chosen for initial comparisons of activity. *unc22* encodes an abundant but nonessential myofilament protein[7–9]. Several thousand copies of *unc-22* mRNA are present in each

Table 1 Effects of sense, antisense and mixed RNAs on progeny of injected animals

Gene	segment	Size (kilobases)	Injected RNA	F_1 phenotype
unc-22				*unc-22*-null mutants: strong twitchers[7,8]
*unc22A**	Exon 21-22	742	Sense	Wild type
			Antisense	Wild type
			Sense + antisense	Strong twitchers (100%)
unc22B	Exon 27	1,033	Sense	Wild type
			Antisense	Wild type
			Sense + antisense	Strong twitchers (100%)
unc22C	Exon 21-22†	785	Sense + antisense	Strong twitchers (100%)
fem-1				*fem-1*-null mutants: femal (no sperm)[13]
fem1A	Exon 10‡	531	Sense	Hermaphrodite (98%)
			Antisense	Hermaphrodite (>98%)
			Sense + antisense	Female (72%)
fem1B	Intron 8	556	Sense + antisense	Hermaphrodite (>98%)
unc-54				*unc-54*-null mutants: paralysed[7,11]
unc54A	Exon 6	576	Sense	Wild type (100%)
			Antisense	Wild type (100%)
			Sense + antisense	Paralysed (100%)§
unc54B	Exon 6	651	Sense	Wild type (100%)
			Antisense	Wild type (100%)
			Sense + antisense	Paralysed (100%)§
unc54C	Exon 1-5	1,015	Sense + antisense	Arrested embryos and larvae (100%)
unc54D	Promoter	567	Sense + antisense	Wild type (100%)
unc54E	Intron 1	369	Sense + antisense	Wild type (100%)
unc54F	Intron 3	386	Sense + antisense	Wild type (100%)
hlh-1				*hlh-1*-null mutants: lumpy-dumpy larvae[16]
hlh1A	Exons 1-6	1,033	Sense	Wild type (<2% lpy-dpy)
			Antisense	Wild type (<2% lpy-dpy)
			Sense + antisense	Lpy-dpy larvae (>90%)‖
hlh1B	Exons 1-2	438	Sense + antisense	Lpy-dpy larvae (>80%)‖
hlh1C	Exons 4-6	299	Sense + antisense	Lpy-dpy larvae (>80%)‖
hlh1D	Intron 1	697	Sense + antisense	Wild type (<2% lpy-dpy)
myo-3-driven GFP transgenes¶				
myo-3::NLS::gfp::lacZ				Makes nuclear GFP in body muscle
gfpG	Exons 2-5	730	Sense	Nuclear GFP-LacZ pattern of parent strain
			Antisense	Nuclear GFP-LacZ pattern of parent strain
			Sense + antisense	Nuclear GFP-LacZ absent in 98% of cells
lacZL	Exon 12-14	830	Sense + antisense	Nuclear GFP-LacZ absent in >95% of cells
myo-3::MtLS::gfp				Makes mitochondrial GFP in body muscle
gfpG	Exons 2-5	730	Sense	Mitochondrial-GFP pattern of parent strain
			Antisense	Mitochondrial-GFP pattern of parent strain
			Sense + antisense	Mitochondrial-GFP absent in 98% of cells
lacZL	Exon 12-14	830	Sense + antisense	Mitochondrial-GFP pattern of parent strain

Each RNA was injected into 6-10 adult hermaphrodites (0.5×10^6–1×10^6 molecules into each gonad arm). After 4-6 h (to clear prefertilized eggs from the uterus), injected animals were transferred and eggs collected for 20-22 h. Progeny phenotypes were scored upon hatching and subsequently at 12-24-h intervals.

* to obtain a semiquantitative assessment of the relationship between RNA dose and phenotypic response, we injected each *unc22A* RNA preparation at a series of different concentrations (see figure in Supplementary information for details). At the highest dose tested (3.6×10^6 molecules per gonad), the individual sense and antisense *unc22A* preparations produced some visible twitching (1% and 11% of progeny, respectively). Comparable doses of double-stranded *unc22A* RNA produced visible twitching in all progeny, whereas a 120-fold lower dose of double-stranded *unc22A* RNA produced visible twitching in 30% of progeny. † *unc22C* also carries the 43-nucleotide intron between exons 21 and 22. ‡ *fem1A* carries a portion (131 nucleotides) of intron 10. § Animals in the first affected broods (layed 4-24 h after injection) showed movement defects indistinguishable from those of *unc-54*-null mutants. A variable fraction of these animals (25%–75%) failed to lay eggs (another phenotype of *unc-54*-null mutants), whereas the remainder of the paralysed animals did lay eggs. This may indicate incomplete interference with *unc-54* activity in vulval muscles. Animals from later broods frequently show a distinct partial loss-of-function phenotype, with contractility in a subset of body-wall muscles. ‖ Phenotypes produced by RNA-mediated interference with *hlh-1* included arrested embryos and partially elongated L1 larvae (the *hlh-1*-null phenotype). These phenotypes were seen in virtually all progeny after injection of double-stranded *hlh1A* and in about half of the affected animals produced after injection of double-stranded *hlh1B* and double-stranded *hlh1C*. A set of less severe defects was seen in the remainder of the animals produced after injection of double-stranded *hlh1B* and double-stranded *hlh1C*). The less severe phenotypes are characteristic of partial loss of function of *hlh-1* (B. Harfe and A.F., unpublished observations). ¶ the host for these injections, strain PD4251, expresses both mitochondrial GFP and nuclear GFP-LacZ (see Methods). This allows simultaneous assay for interference with *gfp* (seen as loss of all fluorescence) and with *lacZ* (loss of nuclear fluorescence). The table describes scoring of animals as L1 larvae. Double-stranded *gfpG* caused a loss of GFP in all but 0-3 of the 85 body muscles in these larvae. As these animals mature to adults, GFP activity was seen in 0-5 additional body-wall muscles and in the 8 vulval muscles. Lpy-dpy, lumpy-dumpy.

striated muscle cell[3]. Semiquantitative correlations between *unc-22* activity and phenotype of the organism have been described[8]: decreases in *unc-22* activity produce an increasingly severe twitching phenotype, whereas complete loss of function results in the additional appearance of muscle structural defects and impaired motility.

Purified antisense and sense RNAs covering a 742-nucleotide segment of *unc-22* had only marginal interference activity, requiring a very high dose of injected RNA to produce any observable effect (Table 1). In contrast, a sense–antisense mixture produced highly effective interference with endogenous gene activity. The mixture was at least two orders of magnitude more effective than either single strand alone in producing genetic interference. The lowest dose of the sense–antisense mixture that was tested, ~60,000 molecules of each strand per adult, led to twitching phenotypes in an average of 100 progeny. Expression of *unc-22* begins in embryos containing ~500 cells. At this point, the original injected material would be diluted to at most a few molecules per cell.

The potent interfering activity of the sense–antisense mixture could reflect the formation of double-stranded RNA (dsRNA) or, conceivably, some other synergy between the strands. Electrophoretic analysis indicated that the injected material was predominantly double-stranded. The dsRNA was gel-purified from the annealed mixture and found to retain potent interfering activity. Although annealing before injection was compatible with interference, it was not necessary. Mixing of sense and antisense RNAs in low-salt concentrations (under conditions of minimal dsRNA formation) or rapid sequential injection of sense and antisense strands were sufficient to allow complete interference. A long interval (>1 h) between sequential injections of sense and antisense RNA resulted in a dramatic decrease in interfering activity. This suggests that injected single strands may be degraded or otherwise rendered inaccessible in the absence of the opposite strand.

A question of specificity arises when considering known cellular responses to dsRNA. Some organisms have a dsRNA-dependent protein kinase that activates a panic-response mechanism[10]. Conceivably, our sense–antisense synergy might have reflected a non-specific potentiation of antisense effects by such a panic mechanism. This is not the case: co-injection of dsRNA segments unrelated to *unc-22* did not potentiate the ability of single *unc-22*-RNA strands to mediate inhibition (data not shown). We also investigated whether double-stranded structure could potentiate interference activity when placed in *cis* to a single-stranded segment. No such potentiation was seen: unrelated double-stranded sequences located 5′ or 3′ of a single-stranded *unc-22* segment did not stimulate interference. Thus, we have only observed potentiation of interference when dsRNA sequences exist within the region of homology with the target gene.

The phenotype produced by interference using *unc-22* dsRNA was extremely specific. Progeny of injected animals exhibited behaviour that precisely mimics loss-of-function mutations in *unc-22*. We assessed target specificity of dsRNA effects using three additional genes with well characterized phenotypes (Fig. 1, Table 1). *unc-54* encodes a body-wall-muscle heavy-chain isoform of myosin that is required for full muscle contraction[7,11,12]; *fem-1* encodes an ankyrin-repeat-containing protein that is required in hermaphrodites for sperm production[13,14]; and *hlh-1* encodes a *C. elegans* homologue of myoD-family proteins that is required for proper body shape and motility[15,16]. For each of these genes, injection of related dsRNA produced progeny broods exhibiting

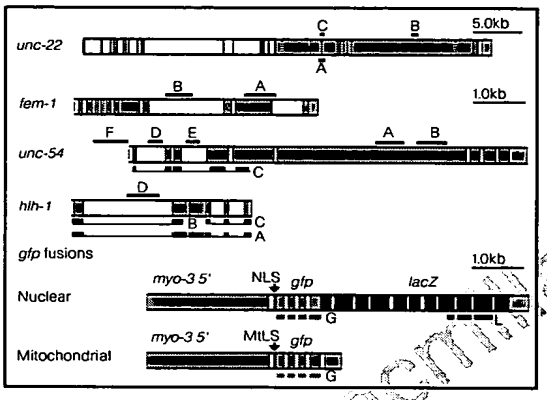

Figure 1 Genes used to study RNA-mediated genetic interference in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (grey and filled boxes, exons; open boxes, introns; patterned and striped boxes, 5′ and 3′ untranslated regions. *unc-22*: ref. 9, *unc-54*, ref. 12, *fem-1*, ref. 14, and *hlh-1*, ref. 15). Each segment of a gene tested for RNA interference is designated with the name of the gene followed by a single letter (for example, *unc22C*). These segments are indicated by bars and upper-case letters above and below each gene. Segments derived from genomic DNA are shown above the gene; segments derived from cDNA are shown below the gene. NLS, nuclear-localization sequence; MtLS, mitochondrial localization sequence.

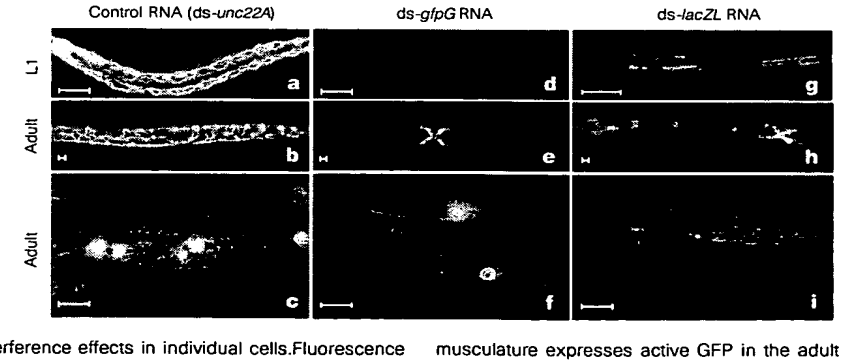

Figure 2 Analysis of RNA-interference effects in individual cells. Fluorescence micrographs show progeny of injected animals from GFP-reporter strain PD4251. **a–c**, Progeny of animals injected with a control RNA (double-stranded (ds)-*unc22A*). **a**, Young larva, **b**, adult, **c**, adult body wall at high magnification. These GFP patterns appear identical to patterns in the parent strain, with prominent fluorescence in nuclei (nuclear-localized GFP-LacZ) and mitochondria (mitochondrially targeted GFP). **d–f**, Progeny of animals injected with ds-*gfpG*. Only a single active cell is seen in the larva in **d**, whereas the entire vulval musculature expresses active GFP in the adult animal in **e**. **f**, Two rare GFP-positive cells in an adult: both cells express both nuclear-targeted GFP-LacZ and mitochondrial GFP. **g–i**, Progeny of animals injected with ds-*lacZL* RNA: mitochondrial-targeted GFP seems unaffected, while the nuclear-targeted GFP-LacZ is absent from almost all cells (for example, see larva in **g**). **h**, A typical adult, with nuclear GFP-LacZ lacking in almost all body-wall muscles but retained in vulval muscles. Scale bars represent 20 μm.

the known null-mutant phenotype, whereas the purified single RNA strands produced no significant interference. With one exception, all of the phenotypic consequences of dsRNA injection were those expected from interference with the corresponding gene. The exception (segment *unc54C* which led to an embryonic- and larval-arrest phenotype not seen with *unc-54*-null mutants) was illustrative. This segment covers the highly conserved myosin-motor domain, and might have been expected to interfere with activity of other highly related myosin heavy-chain genes[17]. The *unc54C* segment has been unique in our overall experience to date: effects of 18 other dsRNA segments (Table 1; and our unpublished observations) have all been limited to those expected from previously characterized null mutants.

The pronounced phenotypes seen following dsRNA injection indicate that interference effects are occurring in a high fraction of cells. The phenotypes seen in *unc-54* and *hlh-1* null mutants, in particular, are known to result from many defective muscle cells[11,16]. To examine interference effects of dsRNA at a cellular level, we used a transgenic line expressing two different green fluorescent protein (GFP)-derived fluorescent-reporter proteins in body muscle. Injection of dsRNA directed to *gfp* produced marked decreases in the fraction of fluorescent cells (Fig. 2). Both reporter proteins were absent from the affected cells, whereas the few cells that were fluorescent generally expressed both GFP proteins.

The mosaic pattern observed in the *gfp*-interference experiments was nonrandom. At low doses of dsRNA, we saw frequent interference in the embryonically derived muscle cells that are present when the animal hatches. The interference effect in these differentiated cells persisted throughout larval growth: these cells produced little or no additional GFP as the affected animals grew. The 14 postembryonically derived striated muscles are born during early larval stages and these were more resistant to interference. These cells have come through additional divisions (13–14 divisions versus 8–9 divisions for embryonic muscles[18,19]). At high concentrations of *gfp* dsRNA, we saw interference in virtually all striated body-wall muscles, with occasional lone escaping cells, including cells born during both embryonic and postembryonic development. The non-striated vulval muscles, which are born during late larval development, appeared to be resistant to interference at all tested concentrations of injected dsRNA.

We do not yet know the mechanism of RNA-mediated interference in *C. elegans*. Some observations, however, add to the debate about possible targets and mechanisms.

First, dsRNA segments corresponding to various intron and promoter sequences did not produce detectable interference (Table 1). Although consistent with interference at a post-transcriptional level, these experiments do not rule out interference at the level of the gene.

Second, we found that injection of dsRNA produces a pronounced decrease or elimination of the endogenous mRNA transcript (Fig. 3). For this experiment, we used a target transcript (*mex-3*) that is abundant in the gonad and early embryos[20], in which straightforward *in situ* hybridization can be performed[5]. No endogenous *mex-3* mRNA was observed in animals injected with a dsRNA segment derived from *mex-3*. In contrast, animals into which purified *mex-3* antisense RNA was injected retained substantial endogenous mRNA levels (Fig. 3d).

Third, dsRNA-mediated interference showed a surprising ability to cross cellular boundaries. Injection of dsRNA (for *unc-22*, *gfp* or *lacZ*) into the body cavity of the head or tail produced a specific and robust interference with gene expression in the progeny brood (Table 2). Interference was seen in the progeny of both gonad arms, ruling out the occurrence of a transient 'nicking' of the gonad

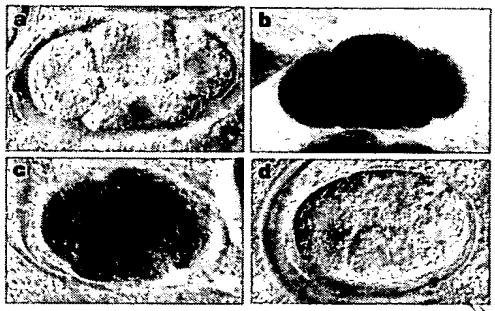

Figure 3 Effects of *mex-3* RNA interference on levels of the endogenous mRNA. Interference contrast micrographs show *in situ* hybridization in embryos. The 1,262-nt *mex-3* cDNA clone[20] was divided into two segments, *mex-3A* and *mex-3B*, with a short (325-nt) overlap (similar results were obtained in experiments with no overlap between interfering and probe segments). *mex-3B* antisense or dsRNA was injected into the gonads of adult animals, which were fed for 24 h before fixation and *in situ* hybridization (ref. 5; B. Harfe and A.F., unpublished observations). The *mex-3B* dsRNA produced 100% embryonic arrest, whereas >90% of embryos produced after the antisense injections hatched. Antisense probes for the *mex-3A* portion of *mex-3* were used to assay distribution of the endogenous *mex-3* mRNA (dark stain). four-cell-stage embryos are shown; similar results were observed from the one to eight cell stage and in the germ line of injected adults. **a**, Negative control showing lack of staining in the absence of the hybridization probe. **b**, Embryo from uninjected parent (showing normal pattern of endogenous *mex-3* RNA[20]). **c**, Embryo from a parent injected with purified *mex-3B* antisense RNA. These embryos (and the parent animals) retain the *mex-3* mRNA, although levels may be somewhat less than wild type. **d**, Embryo from a parent injected with dsRNA corresponding to *mex-3B*; no *mex-3* RNA is detected. Each embryo is approximately 50 μm in length.

Table 2 Effect of site of injection on interference in injected animals and their progeny

dsRNA	Site of injection	Injected-animal phenotype	Progeny phenotype
None	Gonad or body cavity	No twitching	No twitching
None	Gonad or body cavity	Strong nuclear and mitochondrial GFP expression	Strong nuclear and mitochondrial GFP expression
unc22B	Gonad	Weak twitchers	Strong twitchers
unc22B	Body-cavity head	Weak twitchers	Strong twitchers
unc22B	Body-cavity tail	Weak twitchers	Strong twitchers
gfpG	Gonad	Lower nuclear and mitochondiral GFP expression	Rare or absent nuclear and mitochondiral GFP expression
gfpG	Body-cavity tail	Lower nuclear and mitochondrial GFP expression	Rare or absent nuclear and mitochondrial GFP expression
lacZL	Gonad	Lower nuclear GFP expression	Rare or absent nuclear-GFP expression
lacZL	Body-cavity tail	Lower nuclear GFP expresison	Rare or absent nuclear-GFP expression

The GFP-reporter strain PD4251, which expresses both mitochondrial GFP and nuclear GFP-LacZ, was used for injections. The use of this strain allowed simultaneous assay for interference with *gfp* (fainter overall fluorescence), *lacZ* (loss of nuclear fluorescence) and *unc-22* (twitching). Body-cavity injections into the tail region were carried out to minimize accidental injection of the gonad; equivalent results have been observed with injections into the anterior body cavity. An equivalent set of injections was also performed into a single gonad arm. The entire progeny broods showed phenotypes identical to those described in Table 1. This included progeny of both injected and uninjected gonad arms. Injected animals were scored three days after recovery and showed somewhat less dramatic phenotypes than their progeny. This could be partly due to the persistence of products already present in the injected adult. After injection of double-stranded *unc22B*, a fraction of the injected animals twitch weakly under standard growth conditions (10 out of 21 animals). Levamisole treatment led to twitching of 100% (21 out of 21) of these animals. Similar effects (not shown) were seen with double-stranded *unc22A*. Injections of double-stranded *gfpG* or double-stranded *lacZL* produced a dramatic decrease (but not elimination) of the corresponding GFP reporters. In some cases, isolated cells or parts of animals retained strong GFP activity. These were most frequently seen in the anterior region and around the vulva. Injections of double-stranded *gfpG* and double-stranded *lacZL* produced no twitching, whereas injections of double-stranded *unc22A* produced no change in the GFP-fluorescence pattern.

in these injections. dsRNA injected into the body cavity or gonad of young adults also produced gene-specific interference in somatic tissues of the injected animal (Table 2).

The use of dsRNA injection adds to the tools available for studying gene function in *C. elegans*. In particular, it should now be possible functionally to analyse many interesting coding regions[21] for which no specific function has been defined. Although the effects of dsRNA-mediated interference are potent and specific we have observed several limitations that should be taken into account when designing RNA-interference-based experiments. First, a sequence shared between several closely related genes may interfere with several members of the gene family. Second, it is likely that a low level of expression will resist RNA-mediated interference for some or all genes, and that a small number of cells will likewise escape these effects.

Genetic tools are available for only a few organisms. Double-stranded RNA could conceivably mediate interference more generally in other nematodes, in other invertebrates, and, potentially, in vertebrates. RNA interference might also operate in plants: several studies have suggested that inverted-repeat structures or characteristics of dsRNA viruses are involved in transgene-dependent co-suppression in plants[22,23].

There are several possible mechanisms for RNA interference in *C. elegans*. A simple antisense model is not likely: annealing between a few injected RNA molecules and excess endogenous transcripts would not be expected to yield observable phenotypes. RNA-targeted processes cannot, however, be ruled out, as they could include a catalytic component. Alternatively, direct RNA-mediated interference at the level of chromatin structure or transcription could be involved. Interactions between RNA and the genome, combined with propagation of changes along chromatin, have been proposed in mammalian X-chromosome inactivation and plant-gene co-suppression[22,24]. If RNA interference in *C. elegans* works by such a mechanism, it would be new in targeting regions of the template that are present in the final mRNA (as we observed no phenotypic interference using intron or promoter sequences). Whatever their target, the mechanisms underlying RNA interference probably exist for a biological purpose. Genetic interference by dsRNA could be used by the organism for physiological gene silencing. Likewise, the ability of dsRNA to work at a distance from the site of injection, and particularly to move into both germline and muscle cells, suggests that there is an effective RNA-transport mechanism in *C. elegans*. □

Methods

RNA synthesis and microinjection. RNA was synthesized from phagemid clones by using T3 and T7 polymerase[6]. Templates were then removed with two sequential DNase treatments. When sense-, antisense-, and mixed-RNA populations were to be compared, RNAs were further purified by electrophoresis on low-gelling-temperature agarose. Gel-purified products appeared to lack many of the minor bands seen in the original 'sense' and 'antisense' preparations. Nonetheless, RNA species comprising <10% of purified RNA preparations would not have been observed. Without gel purification, the 'sense' and 'antisense' preparations produced notable interference. This interference activity was reduced or eliminated upon gel purification. In contrast, sense-plus-antisense mixtures of gel-purified and non-gel-purified RNA preparations produced identical effects.

Sense/antisense annealing was carried out in injection buffer (ref. 27) at 37 °C for 10–30 min. Formation of predominantly double-stranded material was confirmed by testing migration on a standard (nondenaturing) agarose gel: for each RNA pair, gel mobility was shifted to that expected for dsRNA of the appropriate length. Co-incubation of the two strands in a lower-salt buffer (5 mM Tris-Cl, pH 7.5, 0.5 mM EDTA) was insufficient for visible formation of dsRNA *in vitro*. Non-annealed sense-plus-antisense RNAs for *unc22B* and *gfpG* were tested for RNA interference and found to be much more active than the individual single strands, but twofold to fourfold less active than equivalent preannealed preparations.

After preannealing of the single strands for *unc22A*, the single electrophoretic species, corresponding in size to that expected for the dsRNA, was purified using two rounds of gel electrophoresis. This material retained a high degree of interference activity.

Except where noted, injection mixes were constructed so that animals would receive an average of 0.5×10^6 to 1.0×10^6 RNA molecules. For comparisons of sense, antisense, and double-stranded RNA activity, equal masses of RNA were injected (that is, dsRNA was used at half the molar concentration of the single strands). Numbers of molecules injected per adult are approximate and based on the concentration of RNA in the injected material (estimated from ethidium bromide staining) and the volume of injected material (estimated from visible displacement at the site of injection). It is likely that this volume will vary several-fold between individual animals; this variability would not affect any of the conclusions drawn from this work.

Analysis of phenotypes. Interference with endogenous genes was generally assayed in a wild-type genetic background (N2). Features analysed included movement, feeding, hatching, body shape, sexual identity, and fertility. Interference with *gfp* (ref. 25) and *lacZ* activity was assessed using *C. elegans* strain PD4251. This strain is a stable transgenic strain containing an integrated array (ccIs4251) made up of three plasmids: pSAK4 (*myo-3* promoter driving mitochondrially targeted GFP); pSAK2 (*myo-3* promoter driving a nuclear-targeted GFP–LacZ fusion); and a *dpy-20* subclone[26] as a selectable marker. This strain produces GFP in all body muscles, with a combination of mitochondrial and nuclear localization. The two distinct compartments are easily distinguished in these cells, allowing easy distinction between cells expressing both, either, or neither of the original GFP constructs.

Gonadal injection was done as described[27]. Body-cavity injections followed a similar procedure, with needle insertion into regions of the head and tail beyond the positions of the two gonad arms. Injection into the cytoplasm of intestinal cells is also effective, and may be the least disruptive to the animal. After recovery and transfer to standard solid media, injected animals were transferred to fresh culture plates at 16-h intervals. This yields a series of semisynchronous cohorts in which it was straightforward to identify phenotypic differences. A characteristic temporal pattern of phenotypic severity is observed among progeny. First, there is a short 'clearance' interval in which unaffected progeny are produced. These include impermeable fertilized eggs present at the time of injection. Second, after the clearance period, individuals that show the interference phenotype are produced. Third, after injected animals have produced eggs for several days, gonads can in some cases 'revert' to produce incompletely affected or phenotypically normal progeny.

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Role of the histone deacetylase complex in acute promyelocytic leukaemia

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Non-liganded retinoic acid receptors (RARs) repress transcription of target genes by recruiting the histone deacetylase complex[1–3] through a class of silencing mediators termed SMRT or N-CoR[4,5]. Mutant forms of RARα, created by chromosomal translocations with either the PML (for promyelocytic leukaemia)[6–8] or the PLZF (for promyelocytic leukaemia zinc finger)[9,10] locus, are oncogenic and result in human acute promyelocytic leukaemia (APL). PML–RARα APL patients achieve complete remission following treatments with pharmacological doses of retinoic acids (RA); in contrast, PLZF–RARα patients respond very poorly, if at all[11]. Here we report that the association of these two chimaeric receptors with the histone deacetylase (HDAC) complex helps to determine both the development of APL and the ability of patients to respond to retinoids. Consistent with these observations, inhibitors of histone deacetylase dramatically potentiate retinoid-induced differentiation of RA-sensitive, and restore retinoid responses of RA-resistant, APL cell lines. Our findings suggest that oncogenic RARs mediate leukaemogenesis through aberrant chromatin acetylation, and that pharmacological manipulation of nuclear receptor co-factors may be a useful approach in the treatment of human disease.

Because both PML–RARα and PLZF–RARα inhibit normal retinoid signalling[6–14], we reasoned that identification of factors associated with these proteins might provide mechanistic insights into their oncogenic functions. PLZF–RARα retains the autonomous repression domain, the BTB/POZ (for bric-à-brac/tramtrack/broad complex, poxvirus and zinc-finger) domain, from PLZF[15]. Because deletion of this domain abolishes the biological functions of PLZF–RARα *in vivo*[16,17], we investigated whether it might associate directly with components of the nuclear receptor co-repressor complex[1–3]. By using an *in vitro* interaction assay, we found that radiolabelled full-length mSin3A and histone deacetylase 1 (HDAC1), but not mSin3B, were specifically retained on matrix-bound fusion proteins of glutathione *S*-transferase with the BTB/POZ domain of PLZF (GST–PLZF; Fig. 1a). Results from a yeast two-hybrid assay showed that PLZF interacts with all known components of the co-repressor complex *in vivo* (Fig. 1b). We further mapped the PLZF interaction domain in mSin3A to the paired amphipathic helix 1 (PAH1, residues 112–192) by a mammalian two-hybrid assay (Fig. 1c). Finally, using a co-immunoprecipitation assay from nuclear extracts of transfected CV1 cells, we confirmed that PLZF, SMRT, mSin3A and HDAC1 form a complex in mammalian cells (Fig. 1d). These results, together with the finding that SMRT interacts with another BTB/POZ oncoprotein, LAZ3/BCL6 (ref. 18), demonstrated that this family of transcriptional factors recruits histone deacetylases to repress transcription and implicates histone deacetylases in cellular transformation.

By using a yeast two-hybrid assay, we demonstrated that PLZF–RARα interacts directly with both SMRT and mSin3A, whereas PML–RARα interacts only with SMRT[19] (Fig. 2B). Most importantly, we showed using a co-immunoprecipitation assay that HDAC1 exists in a complex with either PLZF–RARα or PML–RARα in transfected CV1 cells (Fig. 2C). Furthermore, a similar assay using nuclear extracts of the NB4 cells established from a patient with t(15;17) APL[20] indicated that endogenous HDAC1 can be co-precipitated with an anti-PML antibody (Fig. 2D). The presence of endogenous PML–RARα was confirmed by immunoblotting analyses using anti-PML and anti-RARα antibodies (data

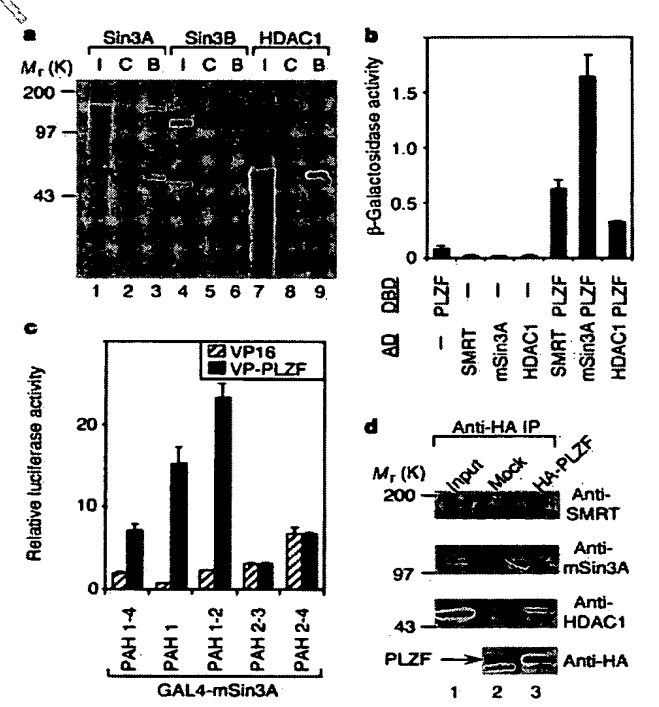

Figure 1 Association of co-repressors and HDAC1 with PLZF. **a**, SDS-PAGE analysis of ^{35}S-labelled mSin3A, mSin3B or HDAC1 proteins retained on immobilized GST-PLZF affinity matrices. I, 20% input; C, GST control; B, bound. **b**, Interactions between PLZF and full-length SMRT, mSin3A or HDAC1 in a yeast two-hybrid assay. **c**, Interactions between GAL4-DBD fusions of different PAHs of mSin3A and VP16 fusion of PLZF are analysed by a mammalian two-hybrid assay in CV1 cells. **d**, PLZF associates with the histone deacetylase complex *in vivo*. CV1 cells were transfected with either vectors only (mock) or plasmids encoding HA-PLZF, SMRT, mSin3A and HDAC1 (HA-PLZF) and nuclear extracts were immunoprecipitated (IP) using anti-HA antibodies followed by immunoblotting analyses using antibodies against SMRT, mSin3A and HDAC1. In lane 1, ~100 μg of nuclear extract was applied to ascertain the positions of blotted proteins (input).

EXHIBIT D

Declaration of Dr. Arthur Riggs

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Myelin Basic Protein Gene and the Function of Antisense RNA in Its Repression in Myelin-Deficient Mutant Mouse

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Abstract: The myelin-deficient (*mld*) mouse is an autosomal recessive mutant characterized by hypomyelination of the CNS due to reduced expression of the myelin basic protein (MBP) gene. In the *mld* mutant, the MBP gene is duplicated in tandem. One gene is intact, but a large portion is inverted upstream of the other copy, and its transcription yields the antisense RNA. This antisense RNA was shown to be localized in the nucleus and to form an RNA:RNA duplex with sense RNA. These findings suggested that inhibition of transport from the nucleus or selective degradation of the duplex is responsible for the reduced expression of the MBP gene in the *mld* mutant. The mechanism of gene rearrangement at the MBP locus was also characterized. Cosmid clones encompassing whole MBP gene loci from control and *mld* genomic DNA libraries were isolated. The recombination points indicated that the duplication and inversion observed in *mld* occurred due to nonhomologous recombination. **Key Words:** Myelin basic protein—Myelin-deficient (*mld*) mutant—RNA:RNA duplex—Gene rearrangement—Antisense RNA. **Okano H. et al.** Myelin basic protein gene and the function of antisense RNA in its repression in myelin-deficient mutant mouse. *J. Neurochem.* **56,** 560–567 (1991).

Myelin is a cellular organelle unique to the nervous system of vertebrate animals. It surrounds neuronal axons in a multilamellar fashion and serves as an insulator, thus facilitating the conduction of neuronal impulses along the axon (reviewed by Ritchie, 1984). Oligodendrocytes are responsible for myelination in the CNS. Myelin basic protein (MBP) is one of the most abundant proteins in CNS myelin (reviewed by Lees and Brostoff, 1984) and is believed to make the myelin compact. Mouse MBP is encoded by a single gene composed of seven exons (de Ferra et al., 1985; Takahashi et al., 1985; Kimura et al., 1986). At least five forms of MBP are produced by alternate use of these seven exons (Newman et al., 1987; Campagnoni, 1988). When expression of the MBP gene is impaired, abnormal myelin lamellae are formed.

Two allelic murine mutants, shiverer (*shi*) (Biddle et al., 1973) and myelin-deficient (*mld*) (Doolittle and Schweikart, 1977), are characterized by abnormal expression of the MBP gene. Studies of *shi* and *mld* have provided insights into the function and mechanism of regulation of MBP in myelination. In *shi*, there is a deletion in the 3′ portion of the gene (from exon 3 to 7) (Roach et al., 1983, 1985; Kimura et al., 1985, 1986), putatively caused by Z-DNA-mediated homologous recombination (Molineaux et al., 1986), and as a result no MBP is produced in *shi*. In *mld*, the MBP gene is duplicated in tandem with some rearrangements (Akowitz et al., 1987; Popko et al., 1987, 1988; Okano et al., 1988*a*) on the distal part of chromosome 18q (Okano et al., 1988*a*). A large portion (including exons 3–7) is inverted in the upstream copy, whereas an intact copy is located downstream in the *mld* locus (Popko et al., 1988). The level of MBP mRNA is reduced in *mld* to ~3% of that of wild-type mRNA (Okano et al., 1986, 1987; Roch et al., 1986; Popko et al., 1987). The reduced mRNA levels, however, are not caused by a mutation within the promoter region (Okano et al., 1988*a*). We demonstrated previously that the upstream copy produces antisense RNA complementary to exons

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Abbreviations used: MBP, myelin basic protein; *mld*, myelin-deficient; *shi*, shiverer.

3 and 7 and proposed that the antisense RNA is responsible for the reduced expression of MBP in the *mld* allele (Okano et al., 1988*b*).

In the present study, we analyzed the mechanism of how antisense RNA represses MBP gene expression by demonstrating the presence of RNA:RNA duplexes and characterizing the intracellular distribution of antisense RNA. In addition, we investigated the process by which the inverted duplication in *mld* is formed. This process appears to involve at least two recombination events, including duplication and inversion, which may have resulted from nonhomologous recombinations.

MATERIALS AND METHODS

Animals

The original pair of *mld* mutants was kindly provided by Dr. N. Baumann. The *mld* mutation had been maintained against a genetic background of MDB/dt. C57Bl/65 mice were used as wild-type controls for analysis of the structure of the MBP gene. ICR mice were used as wild-type controls in the histochemical experiments.

RNase protection studies

Total cellular RNA was isolated from the brains of the *mld* mice and controls according to the method of Chirgwin et al. (1979). Nuclear and cytoplasmic RNA was prepared by the method of Favaloro et al. (1980).

Detection of RNA:RNA duplexes (Fig. 1) was principally based on previously described methods (Kim and Wold, 1985; Melton, 1985). In brief, 20 μg of total cellular RNA samples was digested with RNases under conditions that degrade single-stranded RNA but spare duplexes by incubating the sample with 200 ng of RNase A and 40 units of RNase T1 at 17°C for 30 min. Some of the RNA samples were melted by heating at 100°C for 4 min before the first RNase digestion. RNases were eliminated by incubation with 50 μg of proteinase K at 37°C for 15 min followed by phenol extraction and ethanol precipitation. Remaining RNase-resistant material was combined with aliquots of 5×10^6 cpm of a uniformly ^{32}P-radiolabeled sense RNA probe. The probe detects RNA complementary to exon 3 [probe-2 in the previous article (Okano et al., 1988*b*)]. The subsequent RNase protection experiments were based on the method of the previous article. The undigested RNA probe ($1–2 \times 10^4$ cpm) with a length of 662 bases was used as a molecular weight marker.

Construction of cosmid genomic libraries

Samples of high-molecular-weight DNA (>100 kb) from livers of a wild-type (C57Bl/65) mouse and a homozygous *mld* mouse were partially digested with *Hin*dIII or *Bam*HI, and fragments of ~30–50 kb were isolated on a 10–40% sucrose gradient, inserted into cosmid vector *lorist*B (Cross and Little, 1986) as described by Little (1987), and packaged in vitro (Giga Pack Gold; Stratagene). The *lorist*B was a kind gift of Dr. P. F. R. Little. After adsorption into *Escherichia coli* ED8767 cells, the libraries were screened without amplification using various parts of mouse MBP gene cloned in cos 138 as probes (Takahashi et al., 1985; a kind gift of Dr. L. Hood). Physical maps of cosmid clones were constructed using *cos* mapping technique (λ terminase system and λ mapping system; Amersham).

DNA sequencing

Fragments of DNA to be sequenced were subcloned into pUC19 or pUC119 vectors and sequenced by either the dideoxy-chain termination method of Sanger et al. (1980) or the modified method of Zhang et al. (1988) using the series of 5′-deletion mutants or synthetic oligonucleotide primers.

RESULTS

Antisense RNA duplexes with MBP RNA and localizes in the nucleus

To detect whether antisense *mld* RNA accumulated in the brains of *mld* mice, we used RNase protection analysis with a sense-strand probe corresponding to exon 3 of the *mld* MBP gene. As shown in Fig. 1, the expected 636-base protected fragment of antisense RNA was easily detectable in *mld* mice (lane 3). Antisense RNA was absent in the RNA sample from control mice (lane 4). As a first step toward characterizing the effect of this antisense RNA on MBP gene expression, we determined whether antisense RNA forms RNA:RNA duplexes in vivo. To do this, we first digested total brain cellular RNA with RNases A and T1 under the conditions that degrade single-stranded but not double-stranded RNA. If RNA:RNA duplexes are present, they should be resistant to this first RNase digestion. Next, the RNases were removed, and the RNase-resistant fraction was denatured and hybridized to an uniformly ^{32}P-labeled sense RNA probe that detects RNA complementary to exon 3. After hybridization, the remaining single-stranded probe was digested by RNases A and T1. The final RNase-resistant products were size-fractionated on a sequencing gel.

A discrete band was observed when RNA from *mld* mice was analyzed for RNA:RNA duplex content (Fig. 1, lane 2). The bonafide RNA:RNA duplex would be labile to RNase digestion if it was first heat-denatured. Duplex denaturation by heat pretreatment abolished the 636-base species (data not shown). Therefore, the 636-base species detected in lane 2 of Fig. 1 was con-

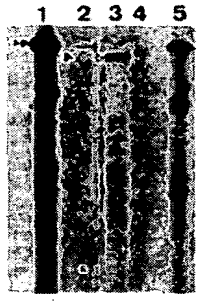


FIG. 1. Detection of RNA:RNA duplexes by RNase protection studies. Total cellular RNA samples (20 μg) from brains of *mld* (lanes 2 and 3) and wild-type control (lane 4) mice were digested with RNases A and T1 under conditions that degrade single-stranded RNA but spare double-stranded RNA (see Materials and Methods). Some RNA samples were hybridized with RNA probe complementary to exon 3 (lanes 2–4). Unhybridized probe was digested with RNases, and the length of the RNase-resistant region of the labeled probe was analyzed by sequencing gel. The presence of RNA:RNA duplexes is indicated (▶) in lane 2. The undigested probe was used as a molecular weight marker of 662 bases (lanes 1 and 5; ▶▶ in lane 1); it includes a polylinker sequence of 10 bases at the 5′ end and 16 bases at the 3′ end. Thus, it is easily distinguishable from the protected band. The RNase protection experiments using total cellular RNA from *mld* (lane 3) and wild-type (lane 4) mice without the first RNase digestion were also performed as controls, based on the method of the previous studies (Okano et al., 1988*b*). The position of the protected band is indicated (▷) in lane 3.

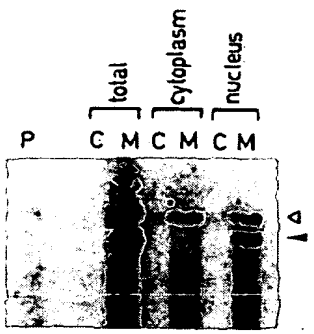


FIG. 2. Subcellular distribution of antisense RNA. Nuclear and cytoplasmic RNAs from control (C) and *mld* (M) mice brains were prepared by the method of Favaloro et al. (1980). Then, RNA samples were characterized by RNase protection experiments with the same RNA probe described in Fig. 1 according to the method previously reported (Okano et al., 1988*b*). The positions of the unhybridized probe (▷) and the protected band (▶) are indicated. P, probe used in this assay.

sidered to be due to the presence of RNA:RNA duplexes rather than incomplete first RNase digestion. From the intensities of bands detected in lanes 2 and 3 of Fig. 1, it was estimated that the steady-state level of RNA:RNA hybrids was <20% of that of the single-stranded antisense RNA. Why this is so is uncertain, but it might be due to greater instability of double-stranded RNA in vivo.

To characterize the intracellular distribution of the antisense RNA, total cellular, cytoplasmic, and nuclear RNA from brains of wild-type control and *mld* mice were analyzed by RNase protection assay (Fig. 2). The antisense RNA is mainly localized in the nuclei of the brains of *mld* mice.

Identification of three recombination points flanking the inverted duplication

To characterize the genetic defect at the *mld* locus, we isolated genomic cosmid clones of the MBP gene from the *mld* and wild-type mice. Together with several λ clones isolated previously [λHOB1011, λHOB1021, and λHOB2031 (Okano et al., 1988*a*)], we were able to obtain a restriction map encompassing the whole MBP locus from wild-type and *mld* mice (85 and 100 kb, respectively; Fig. 3). To determine the junctions of the MBP duplication in the *mld* locus, we compared the *mld* and wild-type restriction maps and DNA sequences and performed Southern blot analyses. There appears to be no major rearrangement within the 14-kb 5′ flanking region of the upstream gene of *mld* on the basis of restriction enzyme mapping (Fig. 3). We found a recombination point 3′ of exon 2 of the upstream gene (referred to as Junction A). To identify the exact recombination point, ~0.3 kb surrounding the recombination points was sequenced in two orientations (Fig. 4). Junction A contained no deletions or additions of nucleotides, although we were unable to determine from which side of Junction A the four nucleotides (TAGA indicated in Fig. 4a) originated. No stretch of homology was detected near the 5′ or 3′ breakage point except for the four nucleotides (TAGA).

Recently, we provided data indicating the location of a recombination point between exon 3 of the upstream gene and exon 1 of the downstream gene. Detailed analysis of λHOB2031, cosHOm12, and cosHOm18-2 in the present study showed that a second recombination point exists between the inverted exon 3 of the upstream gene and exon 1 of the downstream gene. We named the 5′ junction "Junction B" and the 3′ junction "Junction C" (Fig. 4b and 5). The nucleotide sequences surrounding Junction B from normal and *mld* mice are shown in Fig. 4b. The results of physical mapping and nucleotide sequencing analysis surrounding Junctions B and C are also shown in Fig. 5. These results revealed that Junction B is the 3′ boundary of the inverted segment and that Junction C is the 5′ boundary of the downstream gene. A 1,410-bp sequence of unknown origin lies between the two

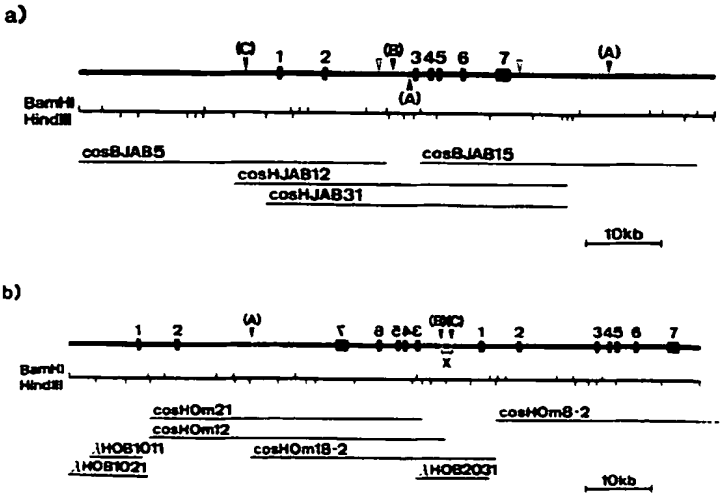


FIG. 3. Genomic organization of the (a) normal and (b) *mld* MBP genes. The positions of cosmid clones characterized in the present study and some λ clones isolated previously (Okano et al., 1988*a*) are presented. Three recombination points (Junctions A, B, and C) are shown (▶). The positions of junctions involved in the *shi* MBP gene (Molineaux et al., 1986) are indicated (▷) in (a). The cleavage sites for *Bam*HI and *Hind*III are also indicated.

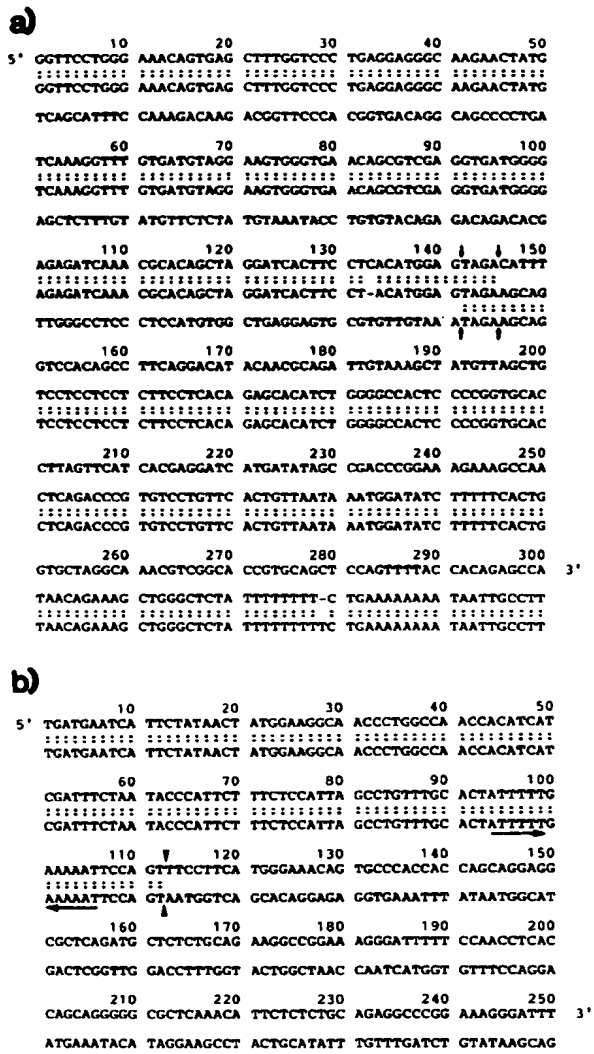


FIG. 4. Nucleotide sequences surrounding the recombination points (Junctions A and B). **a:** Alignment of sequences surrounding Junction A. The top line shows the sequence from the second intron of a normal MBP gene, the middle line shows the sequence from the upstream *mld* MBP gene surrounding Junction A, and the bottom line shows the sequence from the 3' flanking region of a normal MBP gene (complementary strand). **b:** Alignment of sequences surrounding Junction B. Palindromic sequences just 5' to the recombination point are underlined. The top line shows the sequence from the second intron of a normal MBP gene, and the bottom line shows the sequence from the *mld* MBP gene surrounding Junction B.

recombination points (X-region). The X-region did not originate from the normal MBP gene locus (including ~30-kb 5'- and 3'-flanking regions). Genomic Southern hybridization studies revealed that the X-region is repeated in the mouse genome, because there was one band with greater intensity than the single-copy gene (MBP gene in the wild-type control) and smear pattern (data not shown).

It is interesting that the ~2.2-kb DNA sequence at the 3' end of the inverted segment formed an inverted repeat of the 2.2-kb sequence just upstream from Junction A (indicated by the arrows in Figs. 6 and 7).

DISCUSSION

Mechanism of repression of the MBP gene in *mld*

Analysis of the *mld* and *shi* allele has provided insight into the regulation of the MBP gene as well as the function of MBP in the formation of compact myelin lamellae. The organization and regulation of the MBP gene in *mld* are much more complicated than in its null type allele, *shi*. In the normal mouse MBP gene, expression during development is regulated transcriptionally (Carson et al., 1983; Zeller et al., 1984; Okano et al., 1987). The results of transgenic mouse studies by Kimura et al. (1989), in vitro transcription studies by Tamura et al. (1988, 1989*a*,*b*), and transient transfection studies by Miura et al. (1989) indicate that the critical *cis* elements are localized within 1.3 kb (most of them within 120 bp) of the capping site. The function and structure of this promoter region are not impaired in *mld* (Okano et al., 1988*a*). Based on the genomic organization of *mld*, it has been speculated that antisense RNA corresponding to the inverted segment or transcriptional interference might be responsible for the reduced MBP gene expression in *mld* (Okano et al., 1988*b*; Popko et al., 1988). Transcriptional interference is known to be effective in suppressing the 3'-long terminal repeat (3'-LTR) of the integrated retroviral genome (Cullen et al., 1984) and reduced expression of the α-cardiac actin gene in BALB/c mice (Garner et al., 1986). In the case of transcriptional interference, it is believed that the transcript from upstream reaches the transcription initiation site of the downstream gene and inhibits the initiation of transcription. However, transcriptional interference does not seem to contribute significantly to repression of the MBP gene for the following reasons. First, most of the transcript from the upstream gene terminates before reaching the downstream gene (Okano et al., 1988*b*). Second, nuclear run-on assays demonstrated that the transcription rate of the downstream gene is similar to that of the normal MBP gene of the wild-type mice (Roch et al., 1989; Tosic et al., 1989).

The results of the present study indicate that antisense RNA is responsible for repression of the MBP gene, at least in part by forming RNA:RNA duplexes. The antisense RNA is mainly localized in the nucleus and probably inhibits transport of mRNA from the nucleus to the cytoplasm. The results of the present study are consistent with those of Tosic et al. (1989), who showed that nuclear MBP RNA levels are almost normal and correspond to the transcription rate of the downstream gene but that the cytoplasmic RNA level is drastically reduced. All these data indicate that antisense RNA acts mainly posttranscriptionally (by inhibiting transport of mRNA) to repress the MBP gene in *mld*.

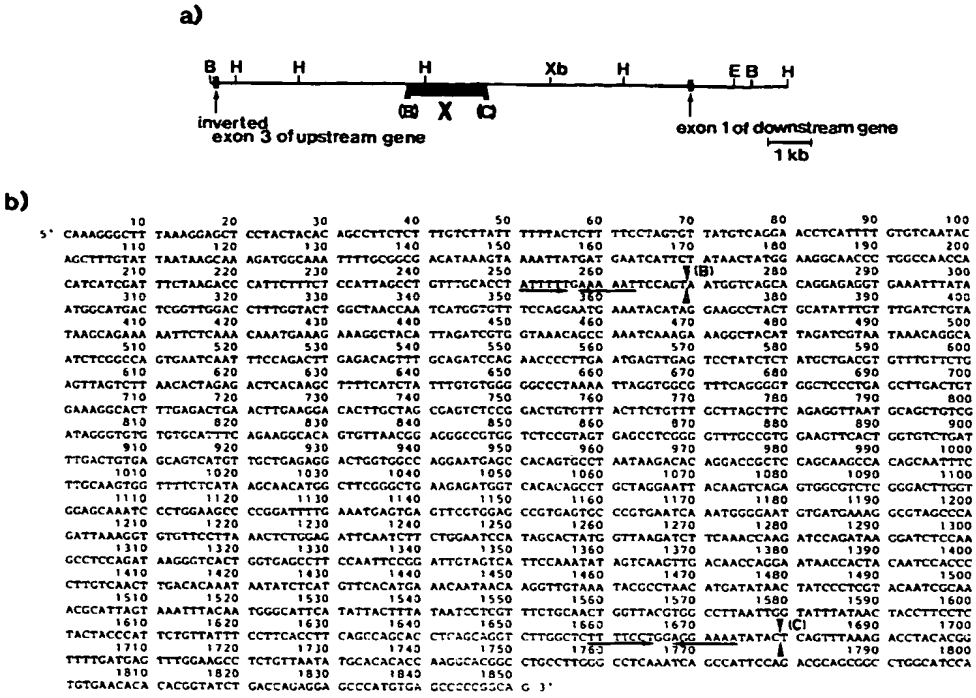


FIG. 5. Physical map (a) and nucleotide sequences (b) of the X-region and surrounding regions. Partial nucleotide sequences near Junction C were derived from our previous results (Okano et al., 1988*b*). In the present studies, we completed the sequencing analysis from Junctions B to C. We named this region the "X-region," as its origin is unknown (see text). Palindromic sequences just 5′ to Junctions B and C are underlined.

Rearrangements in the *mld* locus

The results of the present study indicate that the *mld* locus is not formed by a single recombination event. The gene duplication present in *mld* seems to have arisen by a nonhomologous recombination event, because there is no homology between the sequences around Junction A (3′-flanking sequences) and sequences around Junction C (5′-flanking sequences) of the normal MBP gene. The upstream and downstream genes are not directly joined to each other but are interrupted by an unknown moderately repetitive sequence (X-region). The X-region does not display any significant homology with any of the repetitive sequences or transposons previously reported. It is noteworthy that insertions of unknown sequences associated with DNA recombination are also found in other cases (Botchan et al., 1980; Anderson et al., 1984; Wake et al., 1984).

The mechanism of insertion of the X-region may be similar to the recombination of the immunoglobulin heavy-chain gene switch region. Short stretches of sequences homologous to the sequences found near the recombination points of the immunoglobulin heavy-chain gene switch region are present near Junction C (Okano et al., 1988*b*). It is interesting that we were also able to find palindromic sequences just upstream from Junctions B (ATTTTT-G-AAAAAT) and C (TTTTCCT-GG-AGGAAAA) (Figs. 4a and 5). These sequences and palindromes may have been important to the insertion of the X-region.

The upstream copy of the MBP gene seems to have arisen from a series of a gene duplication and a subsequent inversion. We presume that the upstream copy originated from duplication of the downstream copy. Following this original duplication, the DNA sequences encompassing the upstream exons 3–7 become inverted. This inversion is flanked by 2.2 kb from the middle of the intron 2. These two 2.2-kb sequences flank the upstream inversion in reverse orientation. Thus, these 2.2-kb sequences form inverted repeats. There are at least two models that can account for the formation of the 2.2-kb inverted repeats. In the first model (model 1), inversion seems to be accompanied by DNA replication. Staggered cuts are created at both ends of the 2.2-kb fragment in the second intron (Junctions A and B; see Fig. 3b), and a blunt cut occurs 12.8 kb downstream from exon 7. The 2.2-kb fragment would subsequently be duplicated as a result of filling-in the missing 2.2 kb on both ends of the staggered cut. The inversion is created after rejoining of the segment containing exons 3–7. This inverted fragment includes the duplicated 2.2-kb fragment proximal to exon 3. The palindromic sequence found near Junction B (ATTTT-G-AAAAT; Fig. 4b) might be important in

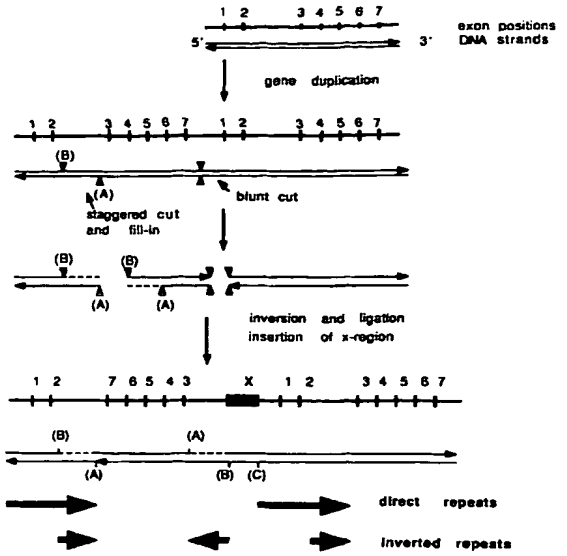


FIG. 6. A model for the reiterated structure of the *mld* locus (model 1). The positions of Junctions A, B, and C are indicated as (A), (B), and (C), respectively. DNA sequences produced by the potential fill-in reaction are indicated by dashed lines. The positions of direct or inverted repeats are also shown by the horizontal arrows at the bottom.

determining the position of the nick in the staggered-cutting step. It is interesting that DNA replication associated with nonhomologous recombination is also found in other cases (Ikeda and Shiozaki, 1984; Denny et al., 1985). In the second model (model 2), the inverted repeat is formed as a result of misalignment of chromosomes during meiosis. At present, we have no data to clarify which model is correct. However, if the first model is correct, the 2.2-kb fragment represents one of the largest examples of staggered-cut and fill-in reactions producing a DNA replication.

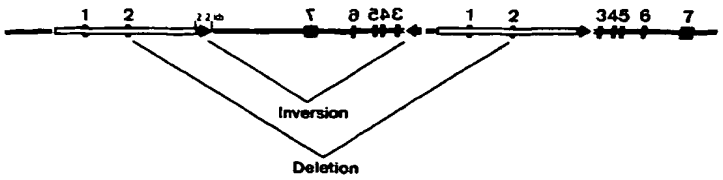
The MBP gene appears to be a hot spot for recombination. It has been shown that the DNA fragment including exons 3–7 is inverted in *mld* mice, whereas it is deleted in *shi* mice (Roach et al., 1985; Kimura et al., 1986; Molineaux et al., 1986). The positions of junctions involved in the rearrangements of *shi* (Molineaux et al., 1986) and *mld* are shown in Fig. 3a. The recombination points within the second intron of *shi* and *mld* (Junction A) are estimated to be ~4 kb apart. The 5′ boundary of the deletion of *shi* and the 3′ boundary of the inversion in *mld* (Junction B) are located very close to each other (~1.4 kb apart).

The high level of recombination associated with the MBP gene might be associated with the presence of Z-DNA. The 3′ side of the deletion in *shi* was mapped to the third of 11 tandem repeats of a 31-bp sequence that is rich in alternating purine–pyrimidine residues and is preceded by a stretch of $(dG\text{-}dT)_{17}$ (Molineaux et al., 1986). The sequences including the 5′ and 3′ ends of the deleted segment exhibit 75% homology (15 of 20). Based on these results, it has been speculated that the purine–pyrimidine tracts, presumably in the Z-DNA form, mediated homologous recombination to form the *shi* locus (Molineaux et al., 1986). Z-DNA is also thought to mediate chromosome translocation (Boehm et al., 1989) and nonhomologous recombination as well as homologous recombination. At present, it is not clear whether the purine–pyrimidine tracts, which are ~11 kb distant from Junction C, are related to the inversion of the *mld* MBP gene.

Biological significance of the genome structure of the *mld* locus: its relation to mosaic expression of MBP

Mosaic expression of MBP in the CNS of *mld* mice has been demonstrated by immunohistochemical studies in situ (Shen et al., 1985; Okano et al., 1986; Mikoshiba et al., 1987). MBP-positive myelins clustered and formed patches in various parts of the CNS of *mld* mice. The amount of MBP in MBP-positive myelin in *mld* is almost the same as in the wild-type control. This mosaic expression has also been also observed in primary cultures of oligodendrocytes (Akowitz et al., 1987). Because it appeared that MBP-positive oligodendrocytes exhibited wild-type levels of MBP expression, these cells were called "revertant" oligodendrocytes (Akowitz et al., 1987). It might be that the "revertant" oligodendrocytes of *mld* result from somatic genetic alteration of the *mld* locus. A 2.2-kb sequence in the second intron was reiterated three times and forms inverted and direct repeats. It seems possible that inversions or deletions occur between these homologous repeats (indicated in Fig. 7). Such potential recombination events are expected to eliminate the inverted segment. This deletion of the segment responsible for repression of the MBP gene in *mld* would enable the oligodendrocytes to produce MBP efficiently. It is also possible that a minor mutation in the duplicated MBP, which would interfere with production of antisense RNA, allows for normal expression in the "revertant" oligodendrocytes. Poly-

FIG. 7. The model of the elimination of the inverted segment in the upstream copy. The 2.2-kb sequences (see text) in the second intron form inverted and direct repeats and are indicated by solid arrows. Long direct repeats formed at 5′ portions of upstream and downstream copies are indicated by open bars. The possible recombinations (inversion or deletion) that eliminate the inverted segment in the upstream copy are also indicated.



merase chain reaction experiments, which are currently in progress in our laboratory, should clarify the genotype of "reverted" oligodendrocytes in *mld*.

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EXHIBIT E

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Antisense RNA: Function and Fate of Duplex RNA in Cells of Higher Eukaryotes

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INTRODUCTION

Numerous examples of naturally occurring antisense RNA-mediated regulation of gene expression in prokaryotes have been documented (140). In most cases the regulation occurs at the translational level (140, 297). The antisense transcript hybridizes to the sense transcript and blocks access of the translational machinery to the 5′ end of the sense transcript. This leads to reduced levels of protein synthesis. On the other hand, cellular compartmentalization in eukaryotes has allowed the development of more numerous and complex effects of antisense transcripts.

There might be some endogenous double-stranded RNA (dsRNA) within the nuclei of most or all eukaryotic cells. The first reports of dsRNA within cells came from studies of heterogeneous nuclear ribonucleoprotein (hnRNP) particles. The presence of highly repetitive sequences within the genome provides the potential for transcripts from different strands to yield RNAs that might anneal. Two groups reported that native RNP particles isolated from HeLa cells might contain 2 to 5% dsRNA, as judged both by nuclease resistance studies and by electron microscopy (42, 85). The majority of the sequences involved in these putative duplexes were reiterated, and the possible physiological significance of these structures was not clear. Since then, there have been many reports of naturally occurring antisense RNA with different modes of action and effects on the cell. The purpose of this review is to briefly summarize some of these reports and to discuss the different ways in which dsRNA can affect gene expression and the ways in which cells deal with the presence of dsRNA. In addition, a major cause of the presence of dsRNA in cells is viral infection. dsRNAs are formed in almost all viral infections and by all types of viruses. Since such molecules have profound effects on cellular physiology, they are reviewed here as well.

Naturally occurring antisense RNAs in higher eukaryotes may be grouped into molecules capable of forming short or imperfect RNA duplexes (less than 100 bp) and molecules capable of forming long, perfect duplexes. Also, examples may be grouped into duplexes in the cytoplasm and duplexes within the nucleus. Each of these scenarios may involve distinct fates for the molecules involved, as well as distinct consequences to the cell.

NATURALLY OCCURRING ANTISENSE RNA IN HIGHER EUKARYOTES

Short (or Imperfect) Duplex RNAs

Although there are a large number of RNA-RNA interactions within cells that are mediated by base pairing between stretches of less than 10 bp of complementary nucleotides, there are only a few examples of regulation of the expression of specific genes by short antisense molecules. These RNAs are generally shorter than 100 bp and are transcribed from a locus that is different from the locus of the sense RNA. Most short-RNA-RNA interactions, such as interactions between small nuclear RNP (snRNP) particle RNAs and pre-mRNA splicing substrates (reviewed in reference 170), as well as the involvement of small nucleolar RNAs in rRNA maturation (299), are beyond the scope of this review. The antisense regulation in each of the two systems described below presumably occurs in the cytoplasm, at the level of translational inhibition.

lin-4/lin-14 of *C. elegans*. In *Caenorhabditis elegans*, antisense RNAs expressed from the *lin-4* gene are complementary to the 3′ end of *lin-14* mRNAs, which are expressed from a different genomic locus. There are two *lin-4* transcripts, of approximately 22 and 61 nucleotides, and they are complementary to the 3′ untranslated region (UTR) of *lin-14* mRNA. *lin-4* transcripts are responsible for the temporal decrease in the levels of Lin-14 protein. In the absence of this regulation (in *lin-4* null mutants) the levels of Lin-14 do not decrease temporally, and this causes a retarded phenotype, namely, the absence of adult structures and the failure to lay eggs (50). *lin-14* transcript levels are constant throughout development, indicating that *lin-14* is negatively regulated posttranscriptionally (330). The antisense RNAs are thought to cause downregulation of Lin-14 expression by interfering with its translation (9, 184, 330). *lin-4* transcripts do not exhibit perfect complementarity to their sense mRNAs but, rather, contain unpaired regions that form bulges that have been shown to be necessary for antisense function (184, 330).

Chicken myosin tcRNA. Another example of regulation by short antisense transcripts is seen in the regulation of the chicken myosin heavy-chain mRNAs by translation control RNAs (tcRNAs) (29, 121–123). tcRNAs, first isolated from embryonic chicken muscle, have complementarity to the 5′ end of chicken myosin heavy-chain mRNAs and inhibit its translation. McCarthy et al. (213) purified myosin heavy-chain mRNP particles from 13-day chicken embryonic skeletal muscle. These authors found a 102-nucleotide tcRNA associated with the mRNPs. tcRNA102 was capable of stoichiometrically inhibiting the translation of the mRNAs with which it associated. Under the same conditions, endogenous reticulocyte mRNA was not inhibited and tcRNA did not associate with rRNA or globin RNA. Like *lin-4* transcripts, tcRNAs do not exhibit perfect complementarity to their target transcripts. As with *lin-4*, the mechanism of action of tcRNA is not known.

Long (or Perfect) Duplex RNAs

Long antisense transcripts are usually transcribed from the same locus as sense RNAs but in the opposite direction and overlapping the region of sense transcription. Williams and

TABLE 1. Some eukaryotic genes with reported complementary transcripts

Gene	Organism	Primary reference(s)
surf-2/surf-4	Mouse	333, 334
	Human	334
bFGF	*Xenopus*	320
	Human	169
		231
	Rat	194
	Chicken	37
erbAα	Human, rat	181
eIF-2α	Human	296
p53	Mouse	73
c-*myc*	Mouse	71
		233
	Human	28
N-*myc*	Human	173
MBP (*mld* mice)	Mouse	243
Igf2r	Mouse	338
H19/Igf2	Mouse	226
BCMA	Human	177
Gart	*Drosophila*	118
Ddc	*Drosophila*	301
PSV-A	*Dictyostelium*	125
GnRH	Rat	6
ERCC1	Human	317
4f-rnp	*Drosophila*	254
Dix-1, Dix-2	*Drosophila*	214
IGF-II	Chicken	308
	Mouse	271
Hoxa 11	Mouse	130
Hsp 70.2	Mouse	229
EPI/PKN	Mouse	19
Ribosomal L27	Mouse	25
c-*myb*	Mouse	26
WT1	Human	43
		314
GnRH	Rat	6
Collagen α1 (I)	Chicken	84

Fried (333) reported the first mammalian example of possible endogenous antisense RNA expression in the mouse surfeit locus (334). Processed mRNAs from two adjacent convergent transcription units, Surf-2 and Surf-4, showed an overlap of 133 bp at their 3′ untranslated ends. The potential interaction of these RNAs to confer regulation was discussed (333). Since that report was published, a number of other examples of convergent transcription units have been described. Some of these have been well studied and suggest antisense regulation, while others merely report the presence of antisense or convergent transcription. We shall first describe the best-studied examples and those that have been shown or are suspected to play regulatory roles. We only briefly mention some other examples of antisense RNA that have not been demonstrated to function in a regulatory manner. Nonmammalian systems are addressed after the mammalian ones. Table 1 provides a list of most reported instances of naturally occurring antisense transcripts in higher eukaryotes. Some of the systems described below have also been reviewed elsewhere (163, 168, 172, 297).

bFGF. Basic fibroblast growth factor (bFGF) is a highly conserved and ubiquitously distributed mitogen. It is overexpressed in glial tumor cells and promotes their unregulated proliferation. The bFGF gene locus is transcribed into a number of mRNA transcripts including an antisense mRNA derived from the opposite DNA strand. Expression of this natural antisense RNA has been implicated in regulation of the bFGF sense mRNA expression and turnover. While screening a *Xenopus laevis* cDNA library, Volk et al. isolated a clone representing a 1.5-kb polyadenylated transcript with an open reading frame coding for an unknown protein of 25 kDa (320). This putative mRNA spanned part of the bFGF exon 3, within the 3′ untranslated region but in the antisense orientation. The region of overlap and complementarity between the sense and antisense RNAs was greater than 900 bp. The sequence organization on the corresponding genomic fragments revealed that the antisense transcript is spliced. Sequence comparison with elongated transcripts from the bFGF gene in human cells revealed that the gene corresponding to the antisense mRNA is evolutionarily conserved. Recently, it has been shown that the long open reading frame within bFGF antisense RNA predicts a hypothetical protein with homology to the prokaryotic MutT family of nucleotide hydrolases (167, 195). Antibodies directed against the conserved MutT domain of the deduced human bFGF antisense protein revealed the expression of an immunoreactive 24-kDa protein in liver extracts from *X. laevis* and two proteins of 28 and 35 kDa in rat liver extracts. The bFGF antisense protein is expressed in a broad range of non-central nervous system (CNS) tissue in the postnatal period of the rat.

Kimelman and Kirschner (164) published data from *Xenopus* microinjection experiments suggesting that the antisense transcript can induce the modification of the mRNA encoding bFGF during maturation of the oocyte, converting almost half of the adenosine residues to inosine in the region of overlap between the sense and antisense transcripts. This suggested the action of dsRNA adenosine deaminase (ADAR) (which is discussed in greater detail below), but the results were most probably incorrect owing to the inadvertent microinjection of the modifying enzyme during the course of the experiments (276).

Borja et al. (37) studied the expression of sense and antisense bFGF transcripts during embryogenesis. The inversely proportional amounts of sense and antisense transcripts suggested a possible regulatory role of the antisense transcripts. Knee et al. (169) used reverse transcription-PCR and Northern hybridization to determine the presence of bFGF and its antisense RNA in unfertilized human oocytes and postnatal differentiated tissues. bFGF and the antisense transcripts were coexpressed in many tissues, with sense transcripts being more abundant than antisense transcripts in half of the tissues examined. Sense and antisense transcript expression was approximately equal in the kidneys and colon, whereas antisense transcripts predominated in the heart, liver, skeletal muscle, and testes.

Murphy and Knee (231) studied bFGF mRNA in human U87-MG glioma cells. They identified the human equivalent of the *Xenopus* antisense transcript and studied its role in bFGF mRNA stability. Analysis of the 3′ UTR of the human bFGF mRNA revealed two areas with greater than 75% homology to exons 3 and 4 of the *Xenopus* antisense transcript. A 1.5-kb antisense transcript was found in normal rat tissues and human T47D breast cancer cells, which contain very low levels of bFGF mRNA. In contrast, antisense transcript expression was undetectable in U87-MG cells, which overexpress the bFGF sense mRNA. The reciprocal relationship between bFGF sense and antisense mRNA expression suggested that antisense transcripts may regulate bFGF expression in mammalian cells and that disruption of normal sense/antisense mRNA ratios may lead to overexpression of bFGF in some tumors.

Li et al. (194) examined the developmental pattern of expression of the bFGF antisense transcript in fetal and postnatal rat tissues. Northern hybridization detected polyadenylated antisense RNA in all tissues examined. Both sense and anti-

sense transcripts were detected in the developing brain, but the pattern of their expression was inversely related. These findings supported the possibility of a regulatory role for the antisense transcript in that tissue. However, there was no evidence for a reciprocal relationship between sense and antisense RNA expression in other tissues examined, indicating that the relationship between sense and antisense RNA expression may be tissue specific, at least for some genes.

Thyroid hormone receptor ErbAα/Rev-ErbAα. The rat *erbAα* locus is associated with two alternatively spliced mRNAs, *erbAα1* and *erbAα2*, which are identical except for their 3'-terminal exons. *erbAα1* mRNA encodes a thyroid hormone receptor, while ErbAα2 encodes a similar protein with an altered ligand binding domain. Lazar et al. (181) isolated a rat cDNA encoding a novel member of the thyroid/steroid hormone receptor. This 56-kDa Rev-ErbAα protein is similar in structure to the *erbAα* product but does not bind thyroid hormone. Rev-ErbAα mRNA is present in many tissues. Interestingly, the mRNA overlaps the c-*erbAα2* mRNA (but not the *erbAα1* mRNA) by 269 bp but in the opposite orientation. The bidirectionally transcribed regions are conserved in the human genes, suggesting an important regulatory function (182). The ratio of *erbAα1* to *erbAα2* is highest in cells expressing high levels of Rev-ErbAα mRNA, leading to the hypothesis that base pairing with Rev-ErbAα blocks the splicing of *erbAα2* mRNA, thereby favoring formation of the nonoverlapping *erbAα1* (180). To test this model, Munroe and Lazar (228) used an in vitro splicing system and demonstrated that antisense transcripts spanning the *erbAα2* 3' splice site could inhibit splicing, consistent with a mechanism in which base pairing with a complementary RNA regulates alternative processing of *erbAα1* and *erbAα2* mRNAs.

Finally, a recent report by Hastings et al. (116) examined the levels of *erbAα* mRNA in numerous B-cell lines representing different stages of differentiation. Expression of Rev-ErbAα was found to correlate strongly with an increase in the ratio of *erbAα1* to *erbAα2* mRNA. The correlation between Rev-ErbAα and *erbAα* mRNA is consistent with negative regulation of *erbAα2* via antisense interactions with the complementary Rev-ErbAα mRNA.

eIF-2α. Resting human peripheral blood T cells synthesize proteins at very low rates and contain very low levels of eukaryotic initiation factor eIF-2α mRNA. During mitogenic activation, the level of eIF-2α mRNA increases greater than 50-fold. This effect has been thought to result mainly from the intranuclear stabilization of the primary transcript (60). Analysis of sequences within the first intron (296) revealed a region with homology to the "initiator" (Inr) sequence first described by Smale and Baltimore (298). This Inr element is positioned about 450 bases downstream of the eIF2α promoter and is oriented in the antisense direction. Deletion or mutation of the Inr element resulted in higher expression from an eIF2α promoter-driven reporter gene. Noguchi et al. (237) used reverse transcription-PCR to demonstrate the presence of overlapping sense and antisense transcripts of the eIF-2α gene in resting and activated human T lymphocytes. These authors further characterized *cis*-acting elements that appear to regulate the antisense Inr. A model for the regulation of eIF-2α expression, which involves the rapid degradation of dsRNA generated by sense and antisense transcription, was presented.

p53. In some cells, the expression of p53 might be regulated at the posttranscriptional level. Mouse F9 embryonal carcinoma stem cells differentiate after treatment with retinoic acid and dibutyryl cyclic AMP. This differentiation process is accompanied by the reduction of stable mRNA for p53, while the transcription rate of this gene is not altered (73). This type of regulation is conserved between chickens and mice and occurs during embryonal development (202). This control seemed to be the result of an induced RNA molecule (160).

Khochbin and Lawrence (159) localized the posttranscriptional regulation of p53 mRNA to the nuclear compartment of the cells. These workers identified a 1.3-kb polyadenylated nuclear RNA molecule and showed that it can anneal to the 5' part of the first intron of the p53 gene but in the antisense orientation. This RNA was subsequently shown to be homologous to intron 1 as a result of a B1 repetitive element in the opposite orientation to one within the p53 first intron. More importantly, perhaps, a longer transcript residing in the nuclear compartment appears to represent an antisense transcript of the entire p53 gene and also appears to accumulate concomitantly with a decrease in sense-strand mRNAs (158).

c-myc. In human, rodent and bovine cells, both strands of the c-*myc* gene are transcribed (28, 71, 165, 233). Sense transcription of the c-*myc* gene uses two major promoters, P1 and P2. In normal growing cells, transcripts initiated from P2 predominate over the ones initiated from P1.

Chang et al. (53) measured the effects of interleukin 3 (IL-3) on c-*myc* locus transcription in the IL-3-dependent pre-B-cell line Ba/F3. They found that IL-3 strongly influenced the relative use of the P1 and P2 promoters and that there was a rapid and reversible drop in the levels of c-*myc* mRNA after IL-3 deprivation, as well as a dramatic change in the relative use of P1 and P2. Interestingly, however, there was little change in the rate of initiation of c-*myc* pre-mRNA. Deprivation of IL-3 led to a large increase in antisense transcription. This correlation suggested a negative regulation of c-*myc* mRNA by antisense transcription, but stable antisense transcripts were not detected. Stable antisense RNAs were detected, however, in c-*myc* genes that were rearranged in murine plasmacytomas, where the oncogene was translocated to an immunoglobulin constant-region gene element (302). The opposite-strand RNAs are chimeric, containing c-*myc* antisense and immunoglobulin sense sequences. Spicer and Sonenshein (302) mapped the 5' ends of the stable chimeric transcripts to a site within intron 2 of the c-*myc* gene and demonstrated that the antisense promoter is functional when linked to a reporter gene in transfection studies.

N-myc. The human N-*myc* gene has bidirectional overlapping transcription units. There are multiple antisense transcripts initiating at various sites within the first intron of the N-*myc* pre-mRNA. Some of these are polyadenylated, and some are nonpolyadenylated (173). The nonpolyadenylated antisense transcripts have 5' ends that are complementary to the 5' ends of the N-*myc* sense mRNA. Interestingly, some of the nonpolyadenylated antisense transcripts were found in the cytoplasmic fraction, where most existed in RNA-RNA duplexes with approximately 5% of the sense N-*myc* mRNA. dsRNA formation appeared to occur only with some of the multiple forms of the N-*myc* mRNA. The transcriptional initiation site of the RNA appeared to play a role in determining this selectivity. The sense-antisense duplexes included sequences from both exon 1 and intron 1, suggesting that dsRNA formation might modulate RNA processing by inhibiting the splicing of intron 1.

Murine myelin basic protein. Myelin-deficient (*mld*) mice are autosomal recessive mutants with hypomyelination of the CNS. Mutant mice express only about 2% of the myelin basic protein (MBP) and cytoplasmic mRNA concentrations present in normal mice. Okano et al. (243) demonstrated that in this mutant the MBP gene has undergone a tandem duplication coupled with an inversion. The upstream gene contains an inversion of exons 3 to 7 of the normal gene and therefore

cannot give rise to mature mRNA and functional protein. However, the upstream gene does express an antisense transcript, which elongates through the inverted segment and past the transcriptional initiation site of the downstream gene. The suggestion was made that the antisense RNA interfered with downstream transcription initiation (243). Tosic et al. (314) reported that while the overall transcription rate of the wild-type MBP gene is normal in these mice (ruling out transcriptional interference as a cause of the defect), the rate of transcription of the inverted upstream gene is even higher. Further, the antisense transcript was shown to be nonpolyadenylated and retained within the nucleus. Thus, the high concentration of nuclear antisense RNA strongly suggested that posttranscriptional regulation occurs in *mld* mice through formation of dsRNA. Mikoshiba et al. (223) and Okano et al. (242) also showed that reduced MBP expression was the result of dsRNA formation within the nucleus and suggested that in this system naturally occurring antisense RNA induces the selective degradation of duplexes or inhibits the nucleocytoplasmic transport of MBP mRNA.

Imprinting. Antisense transcripts have been identified in several imprinted genes. The mouse insulin-like growth factor 2 receptor gene *Igf2r* is expressed only from the maternal chromosome (13). The second intron of *Igf2r* contains a 2-kb CpG island called region 2, which is thought to be an imprinting element (305). This region acquires a maternal imprinting pattern, and the maternal chromosome is methylated in diploid cells in the embryonic and adult stages. Paternal specific repression of *Igf2r* occurs, and in all these cases the presence of an antisense transcript has been observed (338). Unmethylated region 2 is required for the transcription of the antisense transcript but not for the production of the sense transcript. The maternal *Igf2r* makes only the sense transcript and cannot make the antisense RNA since region 2 is methylated in the maternal chromosome.

Multiple imprinted sense and antisense transcripts have also been found in a control region upstream of the imprinted *Igf2* (insulin-like growth factor 2) gene in mice (226). In the mouse *Igf2* gene, the maternal allele is silenced during fetal development. Numerous antisense transcripts, along with sense RNAs, are made from a region upstream of the *Igf2* gene containing a tandem repeat. Imprinting depends upon production of both sense and antisense transcripts as well as parental specific methylation of regions flanking the tandem repeat. Both of these phenomena depend upon the presence of a functional *H19* gene (226). Thus antisense transcripts may play an important role in the process of genomic imprinting.

Nonmammalian Systems

In addition to mammalian systems, several examples of antisense transcription have been reported in lower organisms. Of these, the best studied are the Dopa decarboxylase transcripts of *Drosophila* and the regulation of expression of the *Dictyostelium* PSV-A protein. Regulatory roles have been attributed to the antisense transcripts in both of these systems.

There is a region of overlap between the 3′ termini of a pair of convergent transcription units in the Dopa decarboxylase (*Ddc*) region of *Drosophila melanogaster*. This 88-bp genomic region is associated with the 3′ terminus of the mRNA for the Ddc enzyme and, in the opposite orientation, the 3′ terminus of the adjacent gene, whose function is unknown. An analysis of the temporal and spatial distribution of two transcripts within the organism revealed that levels of the two transcripts appear to be reciprocally regulated (301). Within the testes, where the 3′ transcript is maximally expressed, low levels of Ddc transcript were detected.

The *Dictyostelium discoideum* prespore gene product PSV-A is expressed in a highly regulated fashion during growth and development, although transcription of its gene is constitutive (125). The PSV-A mRNA accumulates only when cells form aggregates and establish the prespore-prestalk pattern. In early development and after stalk disaggregation, the mRNA is unstable. At these times, a 1.8-kb antisense transcript from the same locus is expressed. The antisense transcript does not encode a protein and is regulated by a promoter located within the open reading frame of the PSV-A gene. The reciprocal expression of sense and antisense transcripts suggests a possible antisense control of mRNA stability in this system (125).

A number of other nonmammalian systems have been reported to exhibit antisense transcription. Typical of such results are the *Drosophila melanogaster 4f*-rnp and Gart genes. The *D. melanogaster 4f*-rnp gene makes two alternatively spliced mRNAs, which are especially abundant in the CNS. The gene product contains an RNA recognition motif domain, implicated in RNA binding proteins (39). Several isolated cDNAs from the *4f*-rnp region contained extensive A-to-G changes, suggesting the action of an ADAR on the original mRNA (254). Since the action of this enzyme is highly suggestive of the presence of dsRNA within the nucleus (see below), the implication is that the *4f*-rnp gene is transcribed bidirectionally. This work did not show, however, whether the edited mRNAs are expressed into altered proteins in *Drosophila*, nor was the subcellular location of the edited mRNAs revealed.

The Gart gene of *Drosophila melanogaster* encodes three purine pathway enzymatic activities. Interestingly, a pupal cuticle protein gene was found within the first intron of this gene (118). The intronic gene is encoded on the opposite DNA strand from the purine pathway gene and contains an intron. The cuticle protein gene is expressed primarily over a 3-h period in the abdominal epidermal cells of prepupae that secrete the pupal cuticle, while the sense strand is expressed throughout development. It is not yet known how the expression of each gene might affect the expression of the other.

Conclusion

It is evident from the above discussion that although numerous examples of naturally occurring convergent transcription have been reported so far, a general role for antisense RNA in the regulation of gene expression is not yet firmly established. Future research is required not only to document the existence of more examples of antisense expression but also to carry out detailed mechanistic studies to learn how duplex formation can lead to the myriad of effects reported, including effects on transcription, pre-mRNA processing, mRNA transport, and RNA stability.

DOUBLE-STRANDED RNAs IN VIRUS-INFECTED CELLS

dsRNA formation may occur at some point during the life cycle of most viruses (21–24, 166, 183). Viruses that replicate within the cytoplasm (generally RNA viruses) are thought to generate dsRNA in that compartment. Viruses that express their RNAs in the nucleus (generally DNA viruses) could produce RNAs which form duplexes within the cytoplasm or the nucleus, and in most cases the site of dsRNA formation for these viruses has not been determined.

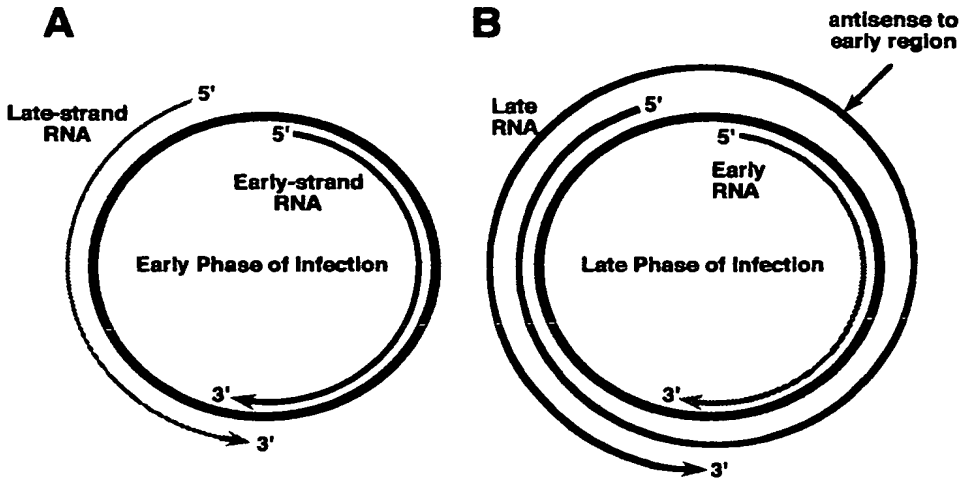

FIG. 1. Temporal regulation of polyomavirus transcript levels. (A) During the early phase of viral infection, early-strand transcripts accumulate preferentially over late-strand transcripts. Late-strand transcripts are processed inefficiently and are relatively unstable. Before DNA replication, the ratio of late-strand to early-strand RNAs is less than 1:10. (B) During the late phase of infection, after the onset of DNA replication, late-strand transcripts are more abundant than early-strand transcripts. Transcription termination is inefficient during this period, allowing RNA polymerase II to encircle the genome multiple times. The resulting multigenomic transcripts contain sequences complementary to early-strand transcripts and act as natural antisense regulators within the nucleus (166). Hatched lines denote transcripts that are downregulated posttranscriptionally.

RNA Viruses

In single-stranded RNA (ssRNA) viruses, the replicative intermediates often consist of dsRNA. For example, by using antisera specific for dsRNA, such molecules were detected in the cytoplasm of cells infected with rubella virus and Semliki Forest virus (183). In dsRNA viruses, the genome itself is the source of dsRNA. However, due to the enormous physiological effects of dsRNAs on the cell, many dsRNA viruses have evolved replication strategies to prevent exposure of dsRNAs to the cytoplasm of cells. In reoviruses, for example, the dsRNA genome remains inside the capsid throughout the viral life cycle and only after the sense strand RNA for progeny viruses is packaged into subviral particles is the second genomic strand synthesized (284). The secondary structure of RNAs may also contribute to the double-stranded nature of RNAs, as seen from human immunodeficiency virus (HIV)- and human T-cell leukemia virus-infected cells (108, 109, 247, 295).

It has been suggested that there might be a role for antisense regulation in the life cycle of HIV-1. Most HIV-1 transcripts from integrated genomes have positive-strand polarity and initiate from the 5' long terminal repeat. However, Michael et al. (222) reported that HIV-1 transcripts with negative-strand polarity could be isolated from acutely and chronically infected cell lines, as well as from peripheral blood mononuclear cell samples from 15 HIV-1-infected patients. Promoter elements critical for negative-strand synthesis were identified and shown to be regulated in a reciprocal fashion to the positive-strand promoter.

DNA Viruses

For DNA viruses, dsRNA arises most often as a result of converging bidirectional transcription. Such RNAs produced from overlapping regions give rise to complementary transcripts. dsRNAs have been found in cells infected by a number of different DNA viruses including adenoviruses (208, 255), herpes simplex virus (141), polyomavirus (4, 76, 106, 309, 316, 329), simian virus 40 (8), and vaccinia virus (36, 62).

The mouse polyomavirus has served as one of the best model systems to study antisense-induced regulation of gene expression. This virus is small and depends heavily on the host for its gene expression. The double-stranded, circular polyomavirus genome is divided into early and late transcription units that are expressed from opposite strands of the viral genome (Fig. 1A). The life cycle of this virus is divided into two phases: the early phase, which occurs immediately after infection and before DNA replication, and the late phase, which begins after the onset of DNA replication. During the early phase of productive infection, early-strand transcripts accumulate preferentially over late-strand transcripts (20, 59, 83, 88, 89, 133, 134, 151, 200, 257). At late times there is a dramatic change in the pattern of gene expression, and the late-strand transcripts are much more abundant than the early-strand transcripts (20, 59, 83, 88, 89, 133, 134, 151, 200, 257). Regulation of both early- and late-strand RNA levels is posttranscriptional (83, 133). During the late phase of infection, RNA polymerase II encircles the genome multiple times (1–3, 5, 32, 83, 134, 315) (Fig. 1B). These giant multigenomic transcripts are the precursors to most late viral mRNAs. Further, these multigenomic RNAs form sense-antisense hybrids with early-strand transcripts (Fig. 1B) and thereby downregulate early-strand mRNA levels (176). Importantly, the antisense portion of the late-strand transcripts is within an intron which is removed during pre-mRNA processing and which remains exclusively within the nucleus. The mechanism of antisense regulation in this system has been elucidated and is described in more detail below.

CELLULAR STRATEGIES TO DEAL WITH CYTOPLASMIC DOUBLE-STRANDED RNA

Double-Stranded RNA Can Induce Interferon

Duplex RNA molecules in the cytoplasm of cells can trigger a profound physiological reaction. As little as a single molecule of dsRNA is sufficient to induce the synthesis of interferon (IFN) (Fig. 2) (209). Since dsRNAs are formed in almost all

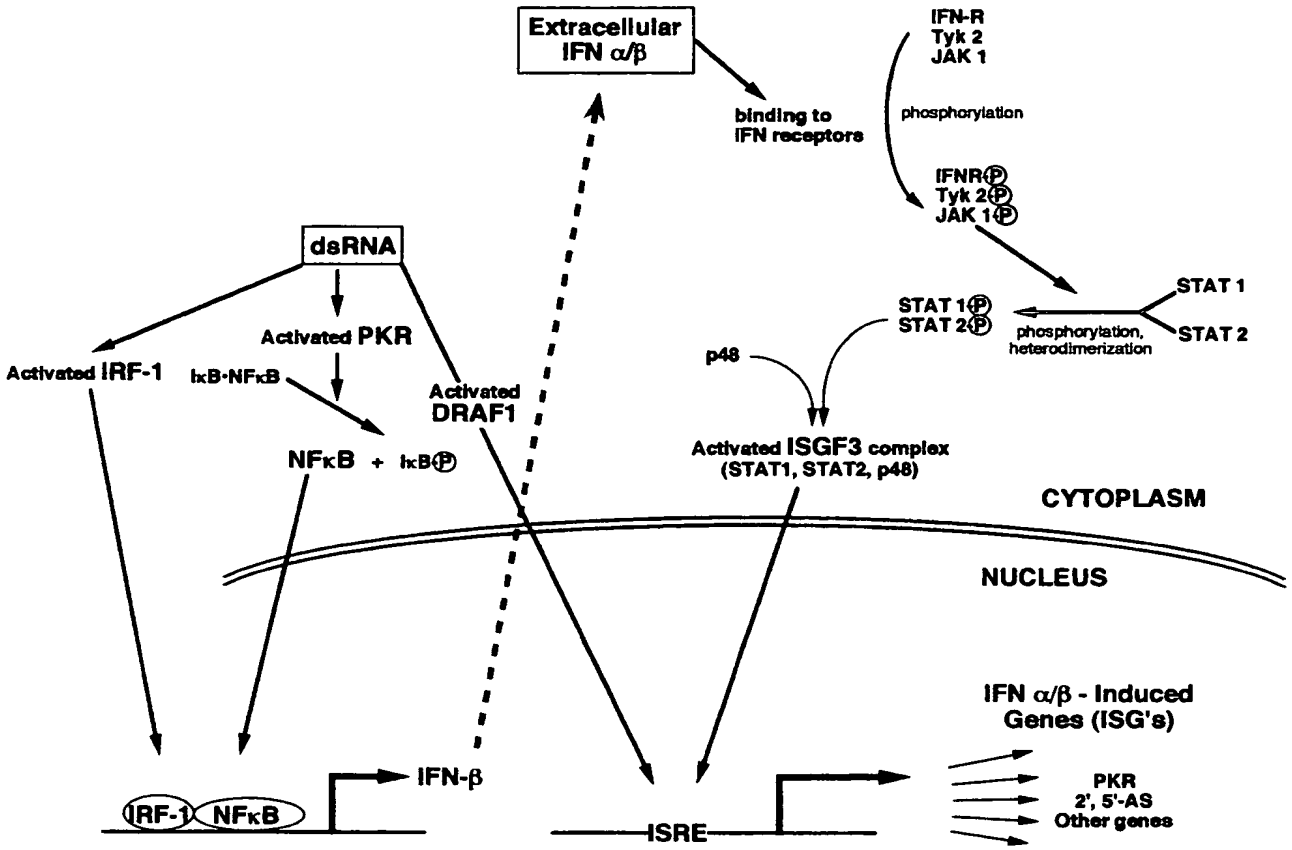

FIG. 2. Signaling pathways of IFN-α, IFN-β, and cytoplasmic dsRNA. The major known pathways of signaling by cytoplasmic dsRNA and IFN-α/β are shown and are discussed in detail in the text. dsRNA directly activates PKR, IRF-1, and DRAF1. PKR phosphorylates IκB, which in turn leads to the nuclear localization of the transcription factor NF-κB. IRF-1 and NF-κB activate the IFN-β promoter, while DRAF1 can activate IFN-α- and IFN-β-induced genes (ISGs). IFN-β is secreted from cells (dashed arrow) and then binds to IFN receptors, leading to the activation of the signal transduction pathway shown, which itself stimulates the expression of ISGs.

viral infections, these can lead to the induction of IFN as well. Interferons are multifunctional cytokines that modulate host immunological functions and can inhibit tumor cell growth and virus multiplication. Most dsRNAs or virus infections induce type I IFNs, which include IFN-α and IFN-β. dsRNA [poly (rI-rC)] specifically induces IFN-β in mouse cells (155). In IFN-treated cells, dsRNA inhibits viral RNA and protein synthesis (reviewed in reference 192). Since the IFN pathways have been reviewed relatively recently (150), we only briefly discuss the most relevant aspects, which are also summarized in Fig. 2.

Important *cis*-Acting Elements in the Alpha and Beta Interferon Promoters

A number of regulatory elements have been identified in the promoter of the IFN-β gene. There are four positive regulatory domains (PRD I to IV) (95, 96, 104, 105) and two negative regulatory domains (NRD I and II) (103, 104, 341). PRD I and III are binding sites for IFN regulatory factor I (IRF-1) (94, 212, 225, 268), PRD II is a binding site for NF-κB (190, 319), and PRD IV is recognized by activating transcription factor 2 (ATF-2/c-Jun) (74, 75). IRF-2 acts as a transcriptional repressor through the NRDs (114). Thanos and Maniatis (311) have suggested that virus induction of IFN-β gene expression in humans requires the assembly of a large complex (an enhanceosome) consisting of IRF-1, NF-κB, ATF-2, and the high-mobility group protein HMG I(Y). HMG I(Y) is not capable of transcriptional activation itself but is required for the binding as well as transcriptional activities of both NF-κB and ATF-2 (281, 311).

Although IFN-β and IFN-γ are produced from single-copy genes, IFN-α is expressed from a family of about 20 genes. All IFN-α genes have a virus-regulated element (VRE), which consists of two sets of repeats; the first contains multiple copies of the pentameric sequence CAGAA, and the other contains repeats of the octameric sequence A(A/T)GGAAAG. Both of these elements are important for virus inducibility of IFN-α (174, 275). The VRE of IFN-α contains a PRD I-like element whose exact sequence determines its ability to be induced by IRF-1 or viruses (206). No NF-κB binding sites are present.

Initiation of the Interferon Pathway by Double-Stranded RNA

A central player in cytoplasmic dsRNA activity is the dsRNA-activated protein kinase (PKR). Cells normally contain basal levels of PKR but in an unphosphorylated, inactive form. When dsRNA is introduced in the cells, PKR binds to and is activated by dsRNA, which induces autophosphorylation. Activated PKR can phosphorylate IκB, which in the unphosphorylated form is complexed with the transcription factor

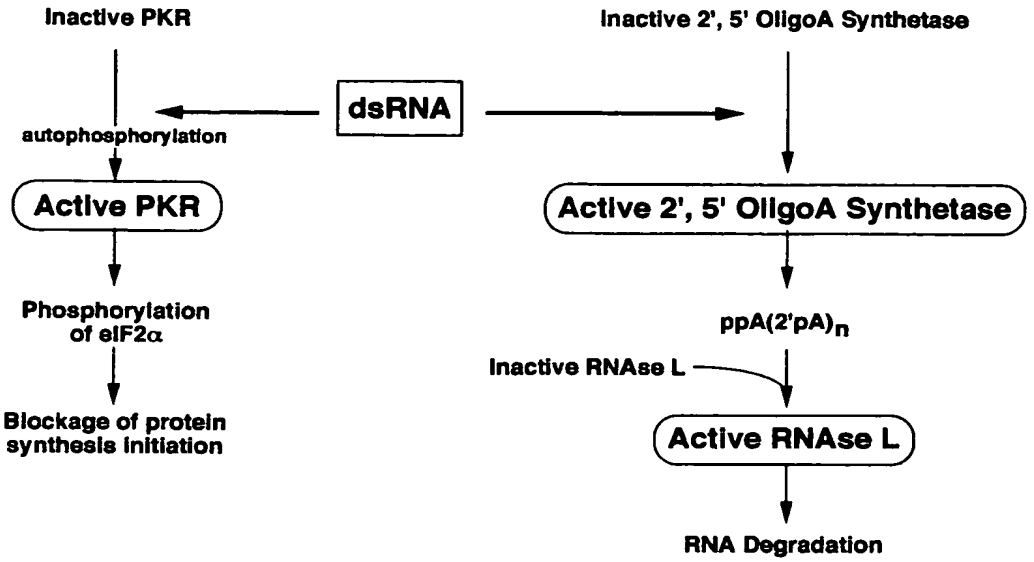

FIG. 3. Cytoplasmic effects of dsRNA. The PKR pathway and the 2',5'-AS/RNase L pathway are directly activated by dsRNA, as described in the text. Activated PKR phosphorylates eIF-2α, which leads to the inhibition of protein synthesis initiation. Activated 2',5'-AS generates oligoadenylates which activate RNase L, which can degrade viral and cellular RNAs.

NF-κB and blocks its nuclear localization signal (175). Upon phosphorylation, IκB is released from NF-κB, which then translocates to the nucleus and activates transcription of genes having NF-κB binding sites. There is an NF-κB site in the PRD II element of the IFN-β gene promoter. dsRNA can also activate the expression of IFNs directly (265, 269, 325). It activates IRF-1, which can bind to the PRD I element present in the promoters of both IFN-α and IFN-β genes and may thus stimulate their expression (96, 256).

Following their synthesis, IFNs are secreted to neighboring cells, where they function as paracrine cytokines and induce a specific class of genes called IFN-stimulated genes (ISGs) (253). The activation of ISGs depends upon the presence of a promoter sequence called the IFN-stimulated response element (ISRE). The ISG factors (ISGFs) bind to this element and activate transcription from these genes.

Signal Transduction Pathway Activated by Interferons

Binding of IFN-α and IFN-β to their transmembrane receptors starts a signal transduction cascade that leads to the expression of ISGs (66, 68, 101, 137, 285). The pathway initiates with the receptor- and ligand-induced phosphorylation of Tyk and JAK kinases (68, 136, 332). Phosphorylated JAK kinases can subsequently phosphorylate signal transducers and activators of transcription (STATs), specifically STAT1 and STAT2 (68, 136). Activated STAT1 and STAT2 dimerize and together form the α subunit of ISGF3 (93, 286). The ISGF3α subunit then associates with the gamma subunit, p48, to form the active ISGF3 complex. This active ISGF3 complex translocates to the nucleus, binds to ISREs, and activates ISGs. A scheme for their mode of action is outlined in Fig. 2. The signal transduction cascades triggered by IFN-α and IFN-β are slightly different from that of IFN-γ, but they induce overlapping sets of genes.

Double-Stranded RNA Can Directly Induce Interferon-Stimulated and Other Genes

In addition to the IFN pathway of ISG induction, dsRNA can directly activate a number of genes, including ISGs (102, 112, 265, 325, 326). Daly and Reich (67) have reported the presence of two dsRNA-activated factors (DRAFs) that bind to the ISRE and flanking sequences and stimulate the expression of these genes directly, independent of the IFN pathway. One of these, DRAF1 (65, 67), is activated by dsRNA. DRAF1 can activate some but not all ISGs (67). Recently, Weaver et al. (328) further characterized the composition of DRAF1. DRAF1 includes IRF-3 and the transcriptional coactivators CREB binding protein and p300. This complex is dependent on dsRNA or viral induction. It has also been shown that both RNA viruses (Newcastle disease virus) and DNA viruses (adenovirus) can activate DRAF1, suggesting that this factor may be used widely for host defense against all types of viruses (65). It has also been reported that IRF-1, activated by dsRNA, can directly stimulate the expression of ISGs (11). Direct stimulation of ISRE-containing genes may be critical for the survival of virus-infected cells because it allows the antiviral effects to occur faster, without having to wait for the synthesis of IFN and the subsequent induction of the antiviral IFN pathway.

A large number of genes contain ISREs and can be activated by IFNs (150). Two of these are key players in pathways that are very important in the inhibition of cellular as well as viral growth. These are the RNA-dependent protein kinase (PKR) pathway and the 2',5'-oligoadenylate synthetase (2',5'-AS)/RNase L pathway (Fig. 3), and each of these is discussed in detail below.

RNA-Dependent Protein Kinase Pathway

A major pathway activated by IFNs and dsRNA is the PKR pathway (Fig. 2 and 3) (reviewed in references 263, 272, 279, 280, and 331). PKR is a serine/threonine kinase that is present in both the nucleus and the cytoplasm. About 20% of PKR

exists in the nucleus (mainly in the nucleoli), and 80% is present in the cytoplasm (144, 145). A total of 80% of the cytoplasmic form of PKR is bound to ribosomes and is more phosphorylated than the nuclear form. PKR is normally present in an inactive or latent state (127, 191, 278) but can be activated by dsRNA.

Activated PKR can phosphorylate a number of substrates, including eukaryotic initiation factor 2α (eIF-2α) (97), IκB (175, 240), HIV Tat (215), and an unidentified cellular 90-kDa protein (270, 312). Phosphorylation of eIF2α has important consequences on cellular translation and can dramatically influence the cell's response to viral infection. PKR phosphorylates eIF-2α at serine residue 51 (277). When phosphorylated, eIF-2α blocks the ability of eIF-2B to catalyze the guanine nucleotide exchange reaction required for protein synthesis initiation (57, 144, 279).

The human (221, 312), mouse (135, 306), and rat (248) PKR genes have been cloned. The PKR enzyme has two important functional domains: an N-terminal RNA binding domain and the C-terminal catalytic domain (279). The N-terminal half of PKR is necessary and sufficient for RNA binding. It binds dsRNA as well as structured ssRNA (87, 107, 154, 248). It does not require a specific sequence for activation and can be activated by very low concentrations (10 to 100 ng/ml) of perfectly duplexed dsRNAs but not by ssRNAs, DNA, RNA-DNA hybrids, or dsRNA duplexes containing modified bases. Short, highly structured viral ssRNAs may inhibit the activation of PKR. At high concentrations, long perfect dsRNAs also inhibit PKR. Activation of PKR by dsRNA most probably results from a conformational change induced by binding of dsRNA. Phosphorylation occurs at several sites within the N-terminal half (307).

Green and Mathews (107) proposed the hinge model for binding of PKR to dsRNA. This model would explain why PKR could bind dsRNAs with such a range of different sizes (22-mers to 80-mers or more). In this model, PKR has two dsRNA binding motifs (dsRBMs) separated by a hinge of about 20 amino acids. A single dsRBM is usually associated with a minimum of 11 bp of dsRNA (41, 157, 287). When dsRBMs bind RNA, the hinge can fold to different angles, to accommodate the binding of RNAs of different sizes. PKR is also known to require dimerization for activation (64, 179, 185, 249, 287, 313). Hunt and Ehrenfeld observed that at high concentrations of dsRNA, PKR is not activated (80, 132). In the dimer model, this would be explained if high concentrations of dsRNAs filled all dsRBMs, preventing two PKR molecules from dimerizing (272).

Some highly structured ssRNAs like the adenovirus VAI RNA can associate with PKR (55) yet can still inhibit its activity (see below). The signal for binding of dsRNA to PKR is the presence of two 2′-hydroxyl groups on the outside of a dsRNA. In highly structured ssRNAs, the two hydroxyl groups might be situated such that they mimic the 2′-hydroxyl groups in authentic dsRNAs (30).

In a recent report, a protein-protein interaction between STAT1 and PKR was shown (335). STAT1 is not a substrate for PKR phosphorylation, but in response to IFNs or dsRNA, the STAT1-PKR complex was dissociated concomitantly with an increase in the DNA binding activity of STAT1. This work suggests that PKR modulates the transcriptional activity of STAT1, which, if true, would further confirm the central role of PKR in both the dsRNA- and IFN-signaling pathways.

2′,5′-Oligoadenylate Synthetase/RNase L Pathway

Another pathway activated by dsRNA and IFNs is the 2′,5′-AS/RNase L pathway (Fig. 3). 2′,5′-AS is activated due to a conformational change that occurs on binding of dsRNA to the enzyme. Activated 2′,5′-AS is capable of polymerizing ATP and other nucleotides in novel 2′,5′ linkages (156). RNase L is activated by these 2′,5′-oligoadenylates. RNase L can cleave both cellular and viral RNAs and specifically cleaves ssRNAs at UpA, UpG, or UpU residues (10, 33, 90, 234, 289, 295, 336). RNase L can also exhibit antiviral effects by inducing apoptosis (45).

There are several distinct 2′,5′-AS activities, with different dsRNA requirements and subcellular locations (54, 138, 273, 282). Thus, they may act locally, to affect different cellular processes. About 70 bp of dsRNA is required for activation (224). The 110-kDa form requires low concentrations of dsRNA for activation and might be responsible for translational effects (128). The 67-kDa form is membrane associated and might affect signal transduction (54, 290). The 40- to 46-kDa nuclear form is associated with the nuclear matrix and activates an RNase that might cleave hnRNA (235).

Like PKR, 2′,5′-AS can bind to dsRNAs or ssRNAs with secondary structure. These substrates include adenovirus VAI RNA (72, 211), reovirus S1 mRNA (34), and HIV Tar RNA (78, 109, 207, 288, 292, 295). The consequences of binding of these RNAs are discussed below.

Other Effects of Double-Stranded RNA on Cells

dsRNA might inhibit protein synthesis in other ways. dsRNA can directly bind to and inactivate eIF-2, but this effect requires rather high intracellular concentrations of dsRNA (115, 149). In avian cells, dsRNA induces the secretion of a nuclease that degrades dsRNA (216). dsRNA sometimes inhibits cell growth in other ways. Low concentrations of dsRNA can inhibit the growth of some tumor cells (91, 318, 342), and this activity is sometimes independent of IFN (131, 196).

Cellular Proteins That Regulate RNA-Dependent Protein Kinase and 2′,5′-Oligoadenylate Synthetase Activities

A number of cellular factors interact with and regulate the activities of PKR and 2′,5′-AS. The most important of these are p58, p67, TAR RNA binding protein (TRBP), and the La autoantigen.

p58. An inhibitor of PKR, p58 is found in human, monkey, bovine, and mouse cells (12, 186–189). p58 binds to PKR directly and inhibits autophosphorylation of PKR as well as phosphorylation of eIF-2α (262). This inhibitor is normally present in an inactive or latent state in the cell because it is complexed with an anti-inhibitor, p52. When p58 and PKR are coexpressed in yeast, p58 prevents PKR activation. However, when p52 was introduced into these strains, PKR activity as well as eIF-2α phosphorylation was restored (98). The anti-inhibitor and inhibitor can be separated by ammonium sulfate fractionation or during influenza virus infection (188). The p58 gene has been cloned and is a member of the tetratricopeptide family of proteins (12, 187). When p58 is overexpressed, cells in culture are transformed, perhaps as a result of an inhibition of PKR (12, 187).

p67. The protein p67 is an eIF-2α-associated cellular protein (69) and can block eIF-2α phosphorylation by PKR (266). Serum starvation of cells leads to degradation of this inhibitor whereas subsequent mitogen stimulation induces its synthesis.

TRBP. Human cellular TRBP (100) binds dsRNA (99, 247) and inhibits the activation of PKR in vitro (247). Overexpres-

sion of TRBP can reverse the effect of the vaccinia virus E3L deletion mutant (247). St. Johnston et al. (304) have identified a homologue of this protein in *X. laevis*. Using an infectious HIV-1 molecular clone, Benkirane et al. (27) have shown that overexpression of TRBP counteracts PKR-mediated inhibition of viral protein synthesis.

La autoantigen. The La autoantigen can also regulate the activity of PKR. La is a 46.7-kDa cellular protein that is located in the nucleus as well as the cytoplasm of eukaryotic cells. It is an RNA binding protein and binds dsRNAs, snRNAs, and certain viral RNAs such as Epstein-Barr virus-encoded small RNAs (EBER) (56, 193) and VAI RNAs of human adenoviruses (92), which are discussed in greater detail below. The La autoantigen can also bind to the internal ribosome entry site of poliovirus transcripts and is required for efficient translation of the viral mRNAs (110, 217, 218). Xiao et al. (339) have demonstrated that the La autoantigen can inhibit dsRNA-dependent autophosphorylation of PKR as well as the ability of PKR to phosphorylate eIF-2α. Excess dsRNA can partially relieve this inhibition. These authors also reported that when the La autoantigen was incubated with synthetic or natural dsRNAs, it could unwind them and convert them into ssRNAs. It is therefore thought that the La autoantigen inhibits PKR activation by binding to dsRNA activators of PKR and converting them into ssRNAs that can no longer activate the kinase.

Other PKR inhibitors. Oncogenic v-*ras* transformation of cells leads to the synthesis of a cellular PKR inhibitor. This 100-kDa inhibitor can act in *trans* to inhibit the auto phosphorylation and hence the activation of PKR (227). Little more is known about this factor. Also, a 15-kDa protein inhibitor of PKR is found in undifferentiated preadipocytes (147, 148). This protein is capable of blocking the interaction of PKR with dsRNA.

VIRAL STRATEGIES TO COUNTERACT HOST DEFENSE MECHANISMS

Many viruses have evolved ingenious strategies to counteract the antiviral effects of IFNs or of dsRNA expressed as a consequence of viral infection. There are a number of examples of this, deriving both from RNA viruses and DNA viruses and from viruses whose replication occurs in the cytoplasm or in the nucleus. Since a major cellular response to viral infections is an increase in the levels of cellular PKR, several viruses have devised strategies to downregulate and/or inactivate this kinase and thus prevent inhibition of protein synthesis. Most viruses use viral proteins or RNAs to downregulate PKR activity, either by direct interactions with PKR, thereby blocking its autophosphorylation and activation, or by sequestration of dsRNA activators of PKR. Other antiviral strategies may include activation of cellular proteins that may either degrade PKR, inhibit its activity, or inhibit the activity of the other major player in the antiviral response, namely, 2′,5′-AS.

DNA Viruses

Adenovirus. VA RNA I is a small (160-nucleotide) viral RNA transcribed by RNA polymerase III and is expressed primarily at the late stages of infection, when it accumulates to very high concentrations (~10^8 molecules/cell) in the cytoplasm (reviewed in reference 210). VA RNA II is a second RNA species, expressed to about ten-fold lower amounts than VA RNA I. Adenovirus mutant *dl*331, which does not synthesize VA RNA I, grows 10-fold less efficiently than the wild type (211). In this mutant, viral protein synthesis is inefficient late in infection. Host protein synthesis is also inhibited. Extracts of *dl*331-infected cells exhibit elevated levels of eIF-2α phosphorylation on serine 51 and reduced levels of guanine nucleotide exchange factor activity (211). In cells containing a serine 51-to-alanine mutation in eIF-2α, the effects of the *dl*331 mutation are suppressed. Therefore, it appears that VA RNA I may prevent the phosphorylation of eIF-2α kinase. VA RNA I acts by binding to PKR and inhibiting its activity. The viral molecule appears to bind PKR via an imperfectly duplexed stem loop structure (107). This binding apparently does not activate PKR because the duplex is too short and imperfect and may prevent the conformational change that occurs during activation when a long duplex RNA binds PKR.

EBV. The human Epstein-Barr virus has two small RNAs, EBER-1 and EBER-2 (193). Like the adenovirus VA RNAs, these are small, are synthesized by RNA polymerase III, and exhibit extensi ve secondary structure (129). In vivo they interact with the La autoantigen (193). EBER-1 interacts with PKR in vitro and can inhibit its kinase activity (58, 81, 294).

Vaccinia virus. Vaccinia virus uses several different mechanisms to inhibit the action of PKR. The viral E3L gene encodes two gene products that can bind dsRNA, sequester it away from PKR, and thus downregulate PKR activity (7, 52, 327, 340). If the E3L gene is deleted, there is a reversal of the kinase inhibitory effect, along with degradation of RNA, which occurs upon activation of the 2′,5′-AS/RNase L pathway (21). Binding of E3L to dsRNA appears to be necessary for vaccinia virus replication in human HeLa cells in culture, as seen in studies with E3L mutants (51). A second gene product of vaccinia virus, K3L, is also capable of inhibiting PKR activity. K3L has 28% identity to eIF-2α but lacks the important phosphorylation site which is found in native eIF-2α subunits (24). The fact that kinase regulation occurs in vivo in vaccinia virus-infected cells has been demonstrated with K3L-defective mutants (24). Therefore, it has been speculated that K3L regulates PKR by associating with PKR and inhibiting its autophosphorylation and activation, thereby preventing phosphorylation of the actual cellular eIF-2α subunit (24, 70, 142).

In addition, vaccinia virus expresses a 57-kDa protein which may inhibit the activity of 2′,5′-AS during viral infection (61, 244, 245). It is thought that p57 inhibits the 2′,5′-AS/RNase L pathway by sequestering its dsRNA activators.

Polyomavirus. Whereas most DNA viruses express gene products (proteins or small RNAs) that interfere with PKR or 2′,5′-AS, the mouse polyomavirus uses a different strategy. Polyomavirus infection induces IFN (76, 106, 309, 316, 329). It has been reported recently that the polyomavirus large T antigen interferes with IFN-inducible gene expression (329). Cell lines derived from wild-type-virus-induced breast tumors are resistant to the growth-inhibitory action of IFN-β and IFN-γ. IFN-induced gene expression is blocked by wild-type virus but not by a mutant that lacks the pRB binding site of the viral large T antigen. The viral large T antigen inhibits IFN-inducible gene expression by binding to JAK-1 kinase and also inhibits the activation of ISGF3 (329). Overexpression of JAK-1 could reverse the IFN-inhibitory effect of the virus (329).

RNA Viruses

Poliovirus. During poliovirus infection, cellular protein synthesis is inhibited at the stage of translation initiation due to proteolytic degradation of a 220-kDa component of eIF-4F (82, 171, 246, 310). eIF-4F is involved in the correct binding of capped mRNA to the 43S initiation complexes (220). Poliovirus mRNAs are uncapped and do not require p220 for its own mRNA translation, because initiation of translation occurs at

an internal ribosome entry site (143). Furthermore, PKR was found to be degraded in poliovirus-infected cells (35). PKR is thought to be degraded by a cellular protease which is activated upon poliovirus infection, and the presence of dsRNA or structured ssRNA is necessary for kinase degradation (152).

Reovirus and influenza viruses. Both reoviruses and influenza viruses use viral proteins to inactivate PKR. Reovirus uses the sigma 3 protein to inhibit PKR. The effect of this inhibition can be reversed by adding large amounts of dsRNA to in vitro reaction mixtures, suggesting that sigma 3 acts by sequestering dsRNA activators of PKR (139).

Influenza virus-infected cells exhibit a dramatic suppression of PKR activity and a decrease in the levels of eIF-2α phosphorylation (153). Moreover, it appears that viral protein synthesis and replication are required for kinase suppression (153). Influenza virus infection activates a 58-kDa, kinase-inhibitory activity, which is actually a cellular protein whose levels remain the same in mock- and virus-infected cells (189). In mock-infected cells, the 58-kDa suppressor protein is present in an inactive state because of its association with an inhibitor of p58 (I-p58). After virus infection, the inhibitor I-p58 may be released from p58. Active p58 is then able to block autophosphorylation of PKR as well as its kinase activity. In addition, the influenza virus NS1 protein can bind dsRNA (117) and PKR activity can be inhibited in vitro by NS1 (203, 264).

HIV. HIV-1 has a stem-loop structure near the 5′ end of viral RNAs called the TAR element. This element can bind PKR (78, 108, 109, 207, 295, 340) and can inhibit its activation by dsRNA (108, 109). The interaction of TAR with PKR can be inhibited by the viral Tat protein (146). It has been reported recently that the viral Tat protein, which binds to the TAR element, not only is a substrate of PKR but also can inhibit it (38).

Conclusion

As should be evident from the above discussion, viruses have devised a wide variety of strategies to counteract host defense mechanisms against duplex RNA. As more viruses are studied in detail, yet more mechanisms or strategies may be revealed. Learning more about the molecular mechanisms by which viruses counteract the dsRNA and IFN systems may not only provide new insights into the underlying mechanisms of cellular responses to duplex RNA but also be of value in the design and implementation of more effective antiviral strategies.

CELLULAR STRATEGIES TO DEAL WITH NUCLEAR DOUBLE-STRANDED RNA

Whereas most viral dsRNAs are thought to be cytoplasmic, most naturally occurring antisense RNA is thought to act within the nucleus (63, 232). It is possible that many cRNAs are expressed within the nucleus, either by design (antisense regulation) or by unintended transcriptional readthrough. As far as is known, dsRNA within the nucleus does not trigger the PKR, IFN, or 2′,5′-AS pathways. What, then, is the fate of dsRNA in this compartment? As discussed above, claims have been made for a variety of effects of antisense RNA, including transcriptional regulation, inhibition of splicing, inhibition of mRNA transport, and induction of mRNA instability. Mechanistically, there are several distinct ways in which nuclear dsRNA molecules might be detected and resolved. They may be degraded by dsRNA-specific nucleases, unwound by dsRNA helicases, or edited by enzymes that modify dsRNAs.

Double-Stranded RNase

Are sense-antisense hybrids the targets of dsRNase activity within the nucleus? Cells might contain a nuclease(s) which is specific for dsRNA. There has been a recent report about the presence of a dsRNase in human cells (337). In this study, chimeric antisense oligonucleotides consisting of 2′-methoxy 5′- and 3′-flanking sequences on either side of an oligoribonucleotide gap were preincubated with sense RNAs and treated with cytosolic or nuclear extracts from T24 human bladder carcinoma cells, and the region of the RNA-RNA hybrid was cleaved. This cleavage was attributed to a dsRNase activity that has been partially purified from these cells. The significance and in vivo activity of such an enzyme is still unclear. However, it should be noted that when dsRNA was microinjected into *Xenopus* oocyte nuclei, it persisted for at least 16 h (16), suggesting that, at least in that system, dsRNAs are not rapidly destroyed within the nucleus.

Helicases

In theory, dsRNAs could be resolved within the cell by the action of helicases that unwind the two strands. A number of proteins which contain intrinsic RNA helicase activity have been identified (reviewed in references 303 and 324). However, there is no evidence that any of these factors acts on long duplexes within the nucleus. Rather, a primary function of such proteins is in the rearrangement of RNA-RNA interactions during RNA processing (303). Importantly, unwinding of duplexes might indeed be a common fate of most or all nuclear antisense RNA interactions. However, this fate might be achieved through the action of another family of enzymes, which are discussed below.

Adenosine Deaminases That Act on Double-Stranded RNA

Figure 4 summarizes what we believe are the consequences and fates of both short and long duplex RNAs formed within the nucleus. The most likely fate of duplex RNA in the nucleus is to be acted upon by a member of the class of enzymes, known as adenosine deaminases, that act on dsRNAs (DRADAs, dsRADs, or, more recently, ADARs [15]). In eukaryotes, a dsRNA unwinding and modifying activity was first discovered in the nucleus of *X. laevis* (16, 267). This enzyme, ADAR1, was subsequently found to be ubiquitous in the animal kingdom (323). ADAR activity is confined almost exclusively to the nucleus, although it was reported recently that a cytoplasmic form of this deaminase may be induced by IFN (198, 199, 250, 251). The first reports were that this enzyme might be a dsRNA-specific unwindase, but this proved not to be the case. ADARs catalyze the conversion of adenosines to inosines within dsRNA (17, 236, 322) by the mechanism of hydrolytic deamination (260). The resultant RNA contains I-U base pairs, which make the RNA duplex unstable and may lead to partial or complete unwinding (17). For ADAR1, the modified adenosines display a 5′-neighbor preference of U > A > C > G (258). The activity of this enzyme may require the presence of a metal ion but does not require any cofactors. In vitro studies suggest that the only substrates for this enzyme are perfectly or imperfectly duplexed RNAs. The activity cannot be competitively inhibited by ssRNA, dsDNA or ssDNA (321).

ADAR1 cDNAs have been cloned from humans (162, 239), rats (239), frogs (126), and, recently, mice (339a), and the protein has been purified from frogs (126), chickens (120), and cows (162, 239). More dsRNA-dependent deaminases have been identified (219), and a family of deaminases is now thought to exist (15). ADAR has been postulated to be in-

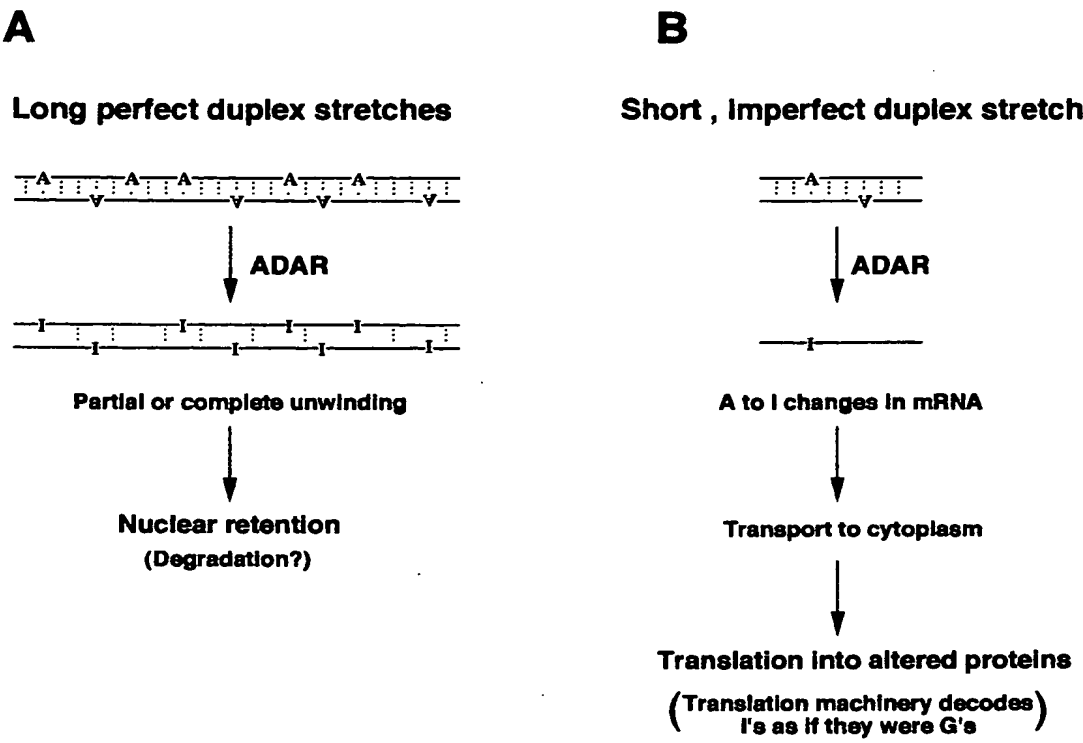

FIG. 4. Nuclear effects of dsRNA. Antisense RNA within the nucleus most probably leads to adenosine modifications by a member of the ADAR family of dsRNA-dependent adenosine deaminases, as discussed in the text. (A) For long duplex stretches, extensive editing occurs. The two RNA strands are partially or fully unwound and are retained exclusively within the nucleus. (B) Short duplex stretches might lead to limited editing, with only one or a few adenosine-to-inosine modifications. Such edited mRNAs can be transported to the cytoplasm, where they are translated.

volved in antisense RNA regulation (14, 161), a view supported by recent results with polyomavirus (reference 176 and discussion below).

ADAR editing of dsRNA is sensitive to the length of the duplex. Duplexes smaller than 15 bp are not modified in vitro (236), and optimal activity is seen with dsRNAs of 100 bp or longer (17, 236). This could be an important property physiologically, and it might help the enzyme discriminate between sequences that are to be modified at a few specific sites and those that are to be extensively edited (such as in antisense RNA regulation). A number of in vivo substrates for ADARs have been described. Most of these have been viral, but several cellular targets are known. A final and interesting characteristic of A-to-I editing is that I's are recognized as if they were G's by the cellular translation machinery. Such a change can never create a translation stop codon, and so all A-to-I changes are missense in character.

Editing of short (or imperfect) duplex regions. (i) Glutamate receptor. Several transcripts of the mammalian glutamate receptor subunits are edited, including gluR-B, gluR-C, gluR-5, and gluR-6 (291). Of these, the first-identified and best-characterized editing is the one found to occur in exon 11 of the gluR-B, gluR-5, and gluR-6 transcripts (124, 300). As a result of the editing, a glutamine codon (CAG) is converted to an arginine codon (CIG). This site is called the Q/R site, and this editing results in an ion channel with altered calcium permeability. Another site which is edited is the R/G site, where an arginine codon (AGA) is converted into a glycine codon (IGA), and this event leads to an ion channel with altered kinetic properties (201). Editing at these sites occurs due to the presence of a double-stranded secondary structure formed by base pairing between an exon and the downstream intron (79, 119, 201). ADAR1 can edit only the R/G site efficiently, and not the Q/R site (205). Another enzyme related to ADAR1, called RED1 or ADAR2, was identified (219). It was subsequently shown by in vitro studies that ADAR2 was capable of editing the Q/R site as well as the R/G site (238). However, the in vivo editing specificities of these enzymes remain to be elucidated. Therefore, there may exist a family of deaminases with each member having overlapping yet distinct specificities. Another member of this family, RED2, has been identified by homology to ADARs (219), and its expression appears to be confined only to the brain, unlike ADAR1 and ADAR2, which are expressed in many tissues.

(ii) Serotonin receptor. Transcripts encoding the 2C subtype of the neurotransmitter serotonin receptor undergo RNA-editing events in which genomically encoded adenosine residues are converted to inosines (40). The serotonin C receptor RNA also appears to be modified by adenosine deamination. These receptors are G-protein-coupled receptors, and the A-to-I editing event in 2C leads to the synthesis of altered proteins in which the interaction between the receptors and the G-proteins is reduced by about 10- to 15-fold (40). Interestingly, in this system, as with the glutamate receptor, editing requires the interaction of exon sequences with downstream intron sequences.

Editing of the HDV antigenomic RNA. RNA editing plays an important role in the hepatitis delta virus (HDV) life cycle. In HDV antigenomic RNA, an amber codon is converted into a codon for tryptophan, thus extending the open reading frame

product by 19 amino acids (44, 178, 204). This editing event most probably occurs by adenosine deamination (259) and is crucial for the viral life cycle because it helps to generate two different protein products from a single viral RNA. Formation of both these proteins is essential for virus multiplication. The smaller form of the delta antigen is required for replication, while the larger protein, formed after RNA editing, represses replication and is required for packaging (178). It has been shown recently that editing of the amber/W site is suppressed by the hepatitis delta antigen (261).

Other examples of endogenous RNA editing. As discussed above, the *4f*-rnp gene in *Drosophila* has been reported to be subject to possible antisense regulation (254) . In this system, cDNAs that contained extensive A-to-I or A-to-G changes were isolated. Also in *Drosophila*, the mRNA for the Para sodium channel in the brain is edited in a fashion similar to that reported for gluR and the serotonin receptor (113).

A-to-I Editing in Long (or Perfect) Duplexes and in Viruses

Adenosine modifications have been seen in a number of RNA viruses. In a persistent measles virus infection, A-to-G as well as U-to-C transitions have been seen (31, 46–49). The cDNA from the matrix gene had about 50% of its U's modified to C's. These U-to-C changes were shown to be the result of adenosine deaminations of the opposite strand (18). Multiple modifications have also been observed in other negative-strand RNA viruses, namely, vesicular stomatitis virus (241), human parainfluenza virus (230), and respiratory syncytial virus (274). Certain retroviral genomes also exhibit modifications. The U3 region of an oncogene c-*mil* transducing retrovirus, IC4, has almost half the A's of its parental Rous sarcoma virus U3 sequence modified to G's (86). The genesis of these mutations is not known but is suggestive of ADAR action. There are two reports of editing in HIV-1. The HIV-1 TAR RNA element shows a single A-to-I mutation, and this change alters viral gene expression (293). Also, Hajjar and Linial (111) described a recombinant HIV-1 provirus generated during in vitro passage that contains a short region of A-to-G hypermutation. The edited region was restricted to complementary sequences present in the recombinant provirus.

In the polyomavirus model system, nuclear antisense RNA leads to both extensive editing and nuclear retention. Work with the polyomavirus model system suggests a mechanism by which long, naturally occurring antisense RNA might function in mammalian cells (176). In polyomavirus-infected mouse NIH 3T3 fibroblasts, natural antisense RNA to viral early-strand transcripts is produced at late times in infection (Fig. 1). This antisense RNA is responsible for the downregulation of viral early-gene expression late in infection. Analysis of early-strand transcripts isolated late in infection revealed extensive base modifications. In many transcripts, about half of the adenosines were altered to inosines or guanosines. Probes that could detect only modified RNAs revealed that these molecules are actually relatively stable and accumulate within the nucleus. Since they are retained in the nucleus, they are inert for gene expression, even though they could theoretically encode mutant viral proteins. Antisense RNA-induced modifications could account for much of the observed regulation, with the lowered levels of early-strand RNAs commonly observed late in infection resulting from the fact that many transcripts are invisible to standard hybridization probes, owing to noncomplementarity. Recent work has shown that extensively edited polyomavirus early-strand RNAs can be polyadenylated and even spliced (176a).

Since polyomavirus is small, the mechanism of antisense RNA regulation observed in this system probably reflects a common mechanism used in all higher eukaryotes. This mechanism involves extensive modifications, leading to RNA "invisibility" to common hybridization probes (perhaps accounting for reports of antisense-induced RNA "decay"). Further, these data lead to the very important conclusion that nuclear antisense RNA leads to nuclear retention of target transcripts.

I-RNase

Scadden and Smith (283) have demonstrated the presence of an RNase in extracts from HeLa cells, sheep uterus, and pig brain that can specifically degrade ssRNAs containing inosines. This enzyme is a 3′-5′ exonuclease that produces 5′ nucleoside monophosphates. The authors suggest that this enzyme is capable of degrading synthetic dsRNAs edited in vitro by the enzyme ADAR2 (RED1). However, the dsRNA substrates edited by ADAR2 were heat denatured to allow the formation of ssRNAs prior to incubation with the enzyme. It has been demonstrated that synthetic dsRNA substrates of ADAR1 that have undergone almost 50% editing of A's to I's are only partially unwound but never completely separated into single strands (17, 322). Since I-RNase is specific for ssRNAs, it is unclear whether the ADAR substrates which never become completely single stranded are degraded by I-RNase. Data obtained Kumar and Carmichael (176) showed that in polyomavirus-infected cells, dsRNAs that have about 50% of the adenosines modified to inosines are maintained stably within the nucleus and can be detected readily even after 6 h of actinomycin D treatment. This suggests that the I-RNase does not act on ADAR substrates in the nuclei of mammalian cells. Moreover, certain substrates of ADARs that have been selectively deaminated at several residues are transported to the cytoplasm stably and are translated to form proteins with altered amino acids e.g., gluR-B. These must also escape degradation by I-RNase in the nucleus as well as the cytoplasm of cells. Further work is required to clarify the role of this interesting enzyme in the cellular response to dsRNA.

Nuclear Antisense RNA Might Generally Induce Adenosine Modifications

As we have seen, most naturally occurring antisense RNA probably acts within the nucleus (63, 232). This would avoid the multitude of physiological effects associated with cytoplasmic dsRNA. The fact that overlapping bidirectional transcription might occur frequently within the nuclei of eukaryotic cells, coupled with the likelihood that long stretches of dsRNA are recognized and extensively modified by ADAR, points to the fact that there may be a novel pool of nuclear RNAs containing inosines (176, 252). Many or most of these RNAs may have been undetected so far because standard hybridization probes will fail to hybridize to them. Inosines have been identified in poly(A)$^+$ mRNAs from various tissues, and it has been found that the levels of inosines correlate with the levels of ADAR expression (252). These data suggest that modification of sense-antisense hybrid RNAs by adenosine deamination may play a very important role in regulating gene expression.

Antisense RNA-mediated gene regulation in eukaryotes has been demonstrated only in the polyomavirus system so far. As seen above, it occurs in the nucleus by ADAR-mediated deamination and nuclear retention of target transcripts. Other eukaryotic antisense systems must be studied in greater detail with the aim of elucidating the nature of antisense regulation and determining whether they also involve ADAR-mediated deamination or some other mechanism(s).

CONCLUSIONS AND FUTURE DIRECTIONS

As we have seen, dsRNA has a number of profound effects on cells, but these effects differ depending on the nature and location of the duplexes. Understanding and appreciating the various pathways of cellular responses to dsRNA will help not only in studies designed to combat viral infections but also in the design and implementation of effective antisense RNA technologies to regulate cellular gene expression.

Finally, is there a wider role for nuclear antisense RNA in the regulation of gene expression? As we have seen, there is increasing evidence for the presence of antisense transcripts associated with the complementary strand of a gene (Table 1). It has been reported that a large fraction of vertebrate mRNAs (perhaps more than 30%) have conserved regions in their 3′ and 5′ UTRs (77). These conserved regions comprise unique sequences in the genome and show sequence conservation only between corresponding regions of orthologous mRNAs in other species. Why is there such strong conservation of these noncoding sequences? Lipman has recently suggested the very interesting model that the long stretches of conserved regions in 3′ UTRs might actually be involved in regulation of RNA stability via the formation of long, perfectly matched sense-antisense duplexes with complementary RNAs (197). In this model, duplexes with perfect matches could be targets of cellular regulatory machinery designed to destabilize or modify the sense transcripts, as a novel mode of regulation. If this is true, antisense RNA regulation would be far more prevalent than has been believed to date.

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EXHIBIT F

Declaration of Dr. Arthur Riggs

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Identification and Partial Purification of Human Double Strand RNase Activity

A NOVEL TERMINATING MECHANISM FOR OLIGORIBONUCLEOTIDE ANTISENSE DRUGS*

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We have identified a double strand RNase (dsRNase) activity that can serve as a novel mechanism for chimeric antisense oligonucleotides comprised of 2′-methoxy 5′ and 3′ "wings" on either side of an oligoribonucleotide gap. Antisense molecules targeted to the point mutation in codon 12 of Harvey Ras (Ha-Ras) mRNA resulted in a dose-dependent reduction in Ha-Ras RNA. Reduction in Ha-Ras RNA was dependent on the oligoribonucleotide gap size with the minimum gap size being four nucleotides. An antisense oligonucleotide of the same composition, but containing four mismatches, was inactive.

When chimeric antisense oligonucleotides were prehybridized with 17-mer oligoribonucleotides, extracts prepared from T24 cells, cytosol, and nuclei resulted in cleavage in the oligoribonucleotide gap. Both strands were cleaved. Neither mammalian nor *Escherichia coli* RNase HI cleaved the duplex, nor did single strand nucleases. The dsRNase activity resulted in cleavage products with 5′-phosphate and 3′-hydroxyl termini.

Partial purification of dsRNase from rat liver cytosolic and nuclear fractions was effected. The cytosolic enzyme was purified approximately 165-fold. It has an approximate molecular weight of 50,000–65,000, a pH optimum of approximately 7.0, requires divalent cations, and is inactivated by approximately 300 mM NaCl. It is inactivated by heat, proteinase K, and also by a number of detergents and several organic solvents.

Antisense oligonucleotides have been shown to inhibit gene expression for a number of cellular targets (1). These compounds have proven to be effective research tools and are of interest as therapeutic agents. To date most antisense oligonucleotides studied have been oligodeoxynucleotides. Oligodeoxynucleotides are believed to cause a reduction in target RNA levels through the action of RNase H (2), an endonuclease that cleaves the RNA strand of RNA:DNA duplexes (3). This enzyme, thought to play a role in DNA replication, has been shown to be capable of cleaving the RNA component of oligodeoxynucleotide:RNA duplexes in cell-free systems as well as in *Xenopus* oocytes (4–6). RNase H is very sensitive to structural alterations in antisense oligonucleotides (7), and thus attempts to increase the potency of oligonucleotides by increasing affinity, stability, lipophilicity, and other characteristics by chemical modifications of the oligonucleotide have often resulted in oligonucleotides that no longer generate substrates for RNase H when bound to their target RNA (8). RNase H activity is also somewhat variable (8), thus a given disease state may not be a candidate for antisense therapy simply because the target tissue has insufficient RNase H activity. Therefore it is clear that terminating mechanisms in addition to RNase H are of potential value to the development of antisense therapeutics.

In addition to the pharmacological inhibition of gene expression described above, it is becoming clear that organisms from bacteria to humans use endogenous antisense RNA transcripts to alter the stability of some target mRNAs and regulate gene expression (9, 10). The best characterized cases of antisense-mediated gene regulation are derived from studies on bacteria; for example an endogenous antisense RNA transcript regulates the expression of *mok* mRNA in certain bacteria. As the antisense RNA level drops, *mok* mRNA levels rise, which leads to the induction of a cytotoxic protein (*hok*), resulting in cell death (11). Other systems regulated by such mechanisms in bacteria include the RNA I-RNA II hybrid of the ColE1 plasmid (12), OOP-cII RNA regulation in bacteriophage λ (13), and the copA-copT hybrids in *Escherichia coli* (14). In *E. coli* the RNA:RNA duplexes formed have been shown to be substrates for regulated degradation by the endoribonuclease RNase III. Duplex-dependent degradation has also been observed in the archaebacterium, *Halobacterium salinarium*, where an antisense transcript reduces expression of the early (T1) transcript of the phage gene *phiH* (15).

In bacteria, RNase III is the double strand endoribonuclease responsible for the degradation of some antisense:sense RNA duplexes. RNase III carries out site-specific cleavage of double strand RNA (dsRNA)[1]-containing structures and also plays an important role in mRNA processing and in the processing of rRNA precursors into 16, 23, and 5 S ribosomal RNA (16). In eukaryotes, a yeast gene (*RNT1*) has recently been cloned that codes for a protein that has striking homology to *E. coli* RNase III and shows dsRNase activity as well as a role in ribosomal RNA processing (17). Avian cells treated with interferon produce and secrete a soluble nuclease capable of degrading dsRNA (18); however, such a secreted dsRNase activity is not a likely candidate to be involved in the intracellular degradation of antisense:sense RNA duplexes. Despite these findings, little is known about human or mammalian dsRNase activities.

In this work we have designed chimeric antisense oligonucleotides that contain 2′-methoxy-modified nucleotides in the "wings" and ribonucleotides in the "gap." These compounds bind to their cellular targets with high affinity to form an oligonucleotide:mRNA duplex in cells. Designing a series of

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[1] The abbreviations used are: ds, double strand; Ha-Ras, Harvey RAS; pCp, cytidine biophosphate.

oligonucleotides with varying ribonucleotide content enabled us to identify, and partially purify, an activity in human cells and rat liver that requires the formation of a dsRNA region (oligoribonucleotide:mRNA) to degrade target RNA in cells. The finding that human cells and rat liver contain an activity capable of recognizing and cleaving dsRNA suggests that human cells may have conserved mechanisms for regulation of gene expression by antisense RNA present in prokaryotes. Further, this activity presents a novel terminating mechanism for antisense drugs. Strategies aiming to exploit this activity to its fullest may have important implications for antisense therapeutics.

MATERIALS AND METHODS

Oligonucleotide Synthesis—RNA gap mer 2′-methoxyphosphorothioate oligonucleotides were synthesized using an Applied Biosystems 380 B automated DNA synthesizer as described previously (19). Oligonucleotides were synthesized using the automated synthesizer and 5′-dimethoxytrityl 2′-tert-butyldimethylsilyl 3′-*O*-phosphoramidite for the RNA portion and 5′-dimethoxytrityl 2′-*O*-methyl 3′-*O*-phosphroamidite for 5′ and 3′ wings. The protecting groups on the exocyclic amines were phenoxyacetyl for riboadenosine and riboguanosine, benzoyl for ribocytosine and 2′-*O*-methyl A and C, and isobutyl for 2′-*O*-methyl G. The standard synthesis cycle was modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2′-methoxy. The fully protected oligonucleotide was cleaved from the support, and the phosphate group was deprotected in 3:1 ammonia/ethanol at room temperature overnight, then lyophilized to dryness. Treatment in methanolic ammonia for 24 h at room temperature was then done to deprotect all bases, and the sample was again lyophilized to dryness. The pellet was resuspended in 1 M tetrabutylammonium fluoride in tetrahydrofuran for 24 h at room temperature to deprotect the 2′ positions. The reaction was then quenched with 1 M triethylaminoacetate, and the sample was then reduced to 0.5 volume by rotovac before being desalted on a G25 size exclusion column (Boehringer Mannheim). The oligonucleotide recovered was then analyzed spectrophotometrically at 260 nm for yield. Purity was characterized by capillary electrophoresis and by mass spectrometry. In all cases the purity was in excess of 90%.

^{32}P Labeling of Oligonucleotides—The sense oligonucleotide was 5′-end-labeled with ^{32}P using [γ-^{32}P]ATP, T4 polynucleotide kinase, and standard procedures (20). The labeled oligonucleotide was purified by electrophoresis on 12% denaturing polyacrylamide gel electrophoresis (20). The specific activity of the labeled oligonucleotide was approximately 5000 cpm/fmol.

Cell Culture and Northern Blot Analysis—T24 human bladder carcinoma cells were maintained as monolayers in McCoys medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and 100 units/ml penicillin. After treatment with oligonucleotide (see below for details) for 24 h, cells were trypsinized and centrifuged, and total cellular RNA was isolated according to standard protocols (20). To quantitate the relative abundance of Ha-Ras mRNA, total RNA (10 μg) was transferred by Northern blotting onto a Bio-Rad Zeta probe membrane (Bio-Rad) and UV cross-linked (Stratalinker, Stratagene, La Jolla, CA). Membrane-bound RNA was hybridized to a ^{32}P-labeled 0.9-kilobase pair Ha-Ras cDNA probe (Oncogene Science, Pasadena, CA) and exposed to XAR film (Eastman Kodak Co.). The relative amount of Ha-Ras mRNA was determined by normalizing the Ha-Ras signal to that obtained when the same membrane was stripped and hybridized with a probe for human glyceraldehyde-3-phosphate dehydrogenase (CLONTECH, Palo Alto, CA). Signals from Northern blots were quantified using a PhosphorImager and Imagequant software (Molecular Dynamics, Sunnyvale, CA).

Oligonucleotide Treatment of Cells—Cells growing as a monolayer were washed once with warm phosphate-buffered saline, then Opti-MEM (Life Technologies, Inc.) medium containing Lipofectin (Life Technologies, Inc.) at a concentration of 5 μg/ml per 200 nM of oligonucleotide up to a maximum concentration of 15 mg/ml was added. Oligonucleotides were added and the cells were incubated at 37 °C for 4 h, after which the medium was replaced with full serum medium. After 24 h in the presence of oligonucleotide, the cells were harvested, and RNA was prepared for further analysis.

RNase H Analysis—RNase H analysis was performed using a chemically synthesized 17-base oligoribonucleotide complementary to bases +23 to +40 of activated (codon 12 mutation) Ha-Ras mRNA. 20 nM of the 5′-end-labeled RNA was incubated with a 100-fold molar excess of the various antisense oligonucleotides in a reaction containing 20 mM Tris-Cl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 4 units of RNase inhibitor (Pharmacia Biotech Inc.) in a final volume of 10 μl. Secondary structures in the oligonucleotides were melted out by heating to 95 °C for 5 min, followed by slow cooling to room temperature. Duplex formation was confirmed by the shift in mobility between the single strand end-labeled sense RNA and the annealed duplex on nondenaturing polyacrylamide gels. The resulting duplexes were tested as substrates for digestion by either *E. coli* RNase HI (U. S. Biochemical Corp., Cleveland, OH) or mammalian RNase HI (partially purified from calf thymus). 1 μl of a 1×10^{-4} mg/ml solution of either *E. coli* RNase HI or mammalian RNase HI was added to 10 μl of the duplex reaction and incubated at 37 °C for 30 min, after which the reaction was terminated by the addition of denaturing loading buffer. Reaction products were resolved on a 12% polyacrylamide gel containing 7 M urea and exposed to XAR film (Kodak).

Cell-free in Vitro Nuclease Assays—Duplexes used in the cell-free T24 extract experiments were annealed as described above. After formation of the duplex the reaction was treated with 1 μl of a mixture of RNase T and A (RPAII kit, Ambion, Austin, TX) and incubated for 15 min at 37 °C, to remove any nonduplexed single strand oligonucleotides. The duplex was then gel-purified from a nondenaturing 12% polyacrylamide gel. T24 cell nuclear and cytosolic fractions were isolated as described previously (21). 10 μl of the annealed duplexes were incubated with 20 μg of the T24 nuclear or cytosolic extract at 37 °C. The reaction was terminated by phenol/chloroform extraction and ethanol-precipitated with the addition of 10 μg of tRNA as a carrier. Pellets were resuspended in 10 μl of denaturing loading dye, and products were resolved on 12% denaturing acrylamide gels as described above. ^{32}P-Labeled 17-base RNA was base-hydrolyzed by heating to 95 °C for 10 min in the presence of 50 mM $NaCO_2$, pH 9.0, to generate a molecular weight ladder.

Duplexes for the rat liver extracts were prepared in 30 μl of reaction buffer (20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.1 mM dithiothreitol) containing 10 nM antisense oligonucleotide and 10^5 cpm of ^{32}P-labeled sense oligonucleotide. Reactions were heated at 90 °C for 5 min and incubated at 37 °C for 2 h. The oligonucleotide duplexes were incubated with either unpurified and semipurified extracts at a total protein concentration of 25 μg of unpurified cytosolic extract, 20 μg of unpurified nuclear extract, 1–4 μl (1–4 μg) ion-exchange-purified cytosolic fraction, or 1–4 μl (100–400 ng) ion-exchange and gel filtration-purified cytosolic fractions or ion-exchange-purified nuclear fraction. Digestion reactions were incubated at 37 °C for 0–240 min. Following incubation, 10 μl of each reaction were removed and quenched by addition of denaturing gel loading buffer (5 μl of 8 M urea, 0.25% xylene cyanol FF, 0.25% bromphenol blue). The reactions were heated at 95 °C for 5 min and resolved in a 12% denaturing polyacrylamide gel. To perform nondenaturing gel analysis, 20 μl of the reaction mixture were quenched by adding 2 μl of the native gel loading buffer (50% glycerol, 0.25% bromphenol blue FF). The reactions were resolved in a 12% native polyacrylamide gel containing 44 mM Tris borate and 1 mM $MgCl_2$. Gels were analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Determination of 5′ and 3′ Termini—Nonlabeled duplex was treated with T24 extracts as described previously. Half of this reaction was then treated with calf intestinal phosphatase (Stratagene) while the other half was left untreated. The phosphatase was inactivated by heating to 95 °C, and the reactions were extracted with phenol/chloroform and then precipitated in ethanol with glycogen as a carrier. The precipitates were then treated with T4 polynucleotide kinase (Stratagene) and [γ-^{32}P]ATP (ICN, Irvine, CA). The samples were again extracted by phenol/chloroform and precipitated with ethanol. The products of the reaction were then resolved on a 12% acrylamide gel and visualized by exposure to Kodak XAR film. The 3′ terminus of the cleaved duplex was evaluated by the reaction of duplex digestion products with T4 RNA ligase (Stratagene) and [^{32}P]pCp (ICN).

Liver Extraction and Preparation of Nuclear and Cytosolic Fractions—0.5 kg of rat liver was blended (Waring Commercial Blender, Dynamics Co. of America, New Hartford, CT) and homogenized (Polytron homogenizer, Brinkmann) in 5 ml of buffer X (10 mM Hepes, pH 7.5, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10% glycerol)/g tissue and centrifuged (Beckman centrifuge J2-21M) at 10,000 rpm for 40 min. The supernatant was precipitated with 40% ammonium sulfate (Sigma). All the activity was recovered in the 40% ammonium sulfate precipitate. The pellet was resuspended in buffer A (20 mM Hepes, pH 6.5, 5 mM EDTA, 1 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride, 0.1 M KCl, 5% glycerol, 0.1% Nonidet P-40, and Triton X-100) and dialyzed to remove ammonium sulfate.

Approximately 40 g of cytosolic extract were obtained from 0.5 kg of liver.

The crude nuclear pellet was resuspended and homogenized in a glass Dounce homogenizer (Tenbroeck Tissue Grinders, Willard, OH) in buffer Y (20 mM Hepes, pH 7.5, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol). The homogenate was centrifuged at 10,000 rpm for 1.5 h. The supernatant was precipitated with 70% ammonium sulfate. The pellet was resuspended and dialyzed in buffer A. Approximately 5 g of nuclear extract were obtained.

Ion-exchange Chromatography—Nuclear and cytosolic extracts in buffer A were centrifuged at 8,000 × *g* for 10 min, and the supernatants were loaded onto Hi-Trap SP ion-exchange (Pharmacia Biotech, Sweden) columns in fast protein liquid chromotography. They were eluted with a linear gradient of NaCl, and samples were collected, directly analyzed for activity, and measured for protein concentration (Bio-Rad).

Gel Filtration High Performance Liquid Chromatography—Active samples from the ion-exchange chromatography were pooled, concentrated by a centrifugal filter device (Millipore Co., Bedford, MA), applied to a TSK G-3000 column (Toso Haas, Montgomeryville, PA) with running buffer A containing 100 mM NaCl. Samples were collected and UV absorption at 280 nM was determined; then they were directly analyzed for activity and measured for protein concentration. Concentrated fractions from the gel filtration chromatography were subjected to 12% SDS-polyacrylamide gel electrophoresis (20).

RESULTS

Chimeric 2'-Methoxy-Oligoribonucleotides (RNA GAP Mer) Mediate Digestion of Target RNA in T24 Cells—In two previous publications, structure-activity analyses of antisense oligonucleotides specific for codon 12 of the Ha-*ras* oncogene containing various 2'-sugar modifications were reported (22, 23). Although the 2'-modified oligonucleotides hybridized with greater affinity to RNA than did unmodified oligodeoxynucleotides, they were completely ineffective in inhibiting Ha-*ras* gene expression (23). The lack of activity observed with these 2'-modified oligonucleotides was attributed to their inability to create duplexes that could serve as substrates for degradation by RNase H when bound to their target RNAs (22). Because 2'-modified, and more specifically, 2'-methoxy oligonucleotides do not result in the nucleolytic degradation of their target mRNA, they provide a unique tool for the identification of novel nucleolytic activities that become activated when structural changes are introduced to fully modified 2'-methoxy antisense oligonucleotides.

In this study we have introduced ribonucleotide stretches of various lengths into the center of 17-base 2'-methoxy oligonucleotides targeting Ha-Ras mRNA, to form 2'-methoxy-ribonucleotide 2'-methoxyphosphorothioate oligonucleotides (RNA gap mers) (see Fig. 1, *A* and *B*, for structures). When hybridized to the cellular mRNA target, the resulting duplex consists of two regions that are not targets for nucleolytic degradation (the 2'-methoxy "wings") and one oligoribonucleotide:RNA duplex region that is potentially a target for a ribonuclease activity that recognizes RNA:RNA duplexes.

T24 human bladder carcinoma cells contain an activating G213T transversion mutation in the Ha-*ras* gene at the codon 12 position (24). Chimeric RNA gap mer antisense oligonucleotides specific for this mutation were transfected into T24 cells growing in culture. After incubation with oligonucleotides for 24 h, cells were harvested, total cytosolic RNA was isolated, and Northern blot analysis for Ha-Ras mRNA levels was performed. As previously observed, fully modified 2'-methoxy oligonucleotides did not support nucleolytic cleavage of target mRNA and therefore did not lead to a reduction in steady state levels of Ha-Ras mRNA, even at the highest concentration tested (Fig. 2*A*, *top panel*, *full 2'-methoxy*). An RNA gap mer oligonucleotide with only 3 ribonucleotides in the gap was also incapable of inducing nucleolytic cleavage of the target RNA (Fig. 2*A*, *bottom panel*, *3 GAP RNA*). However, T24 cells treated

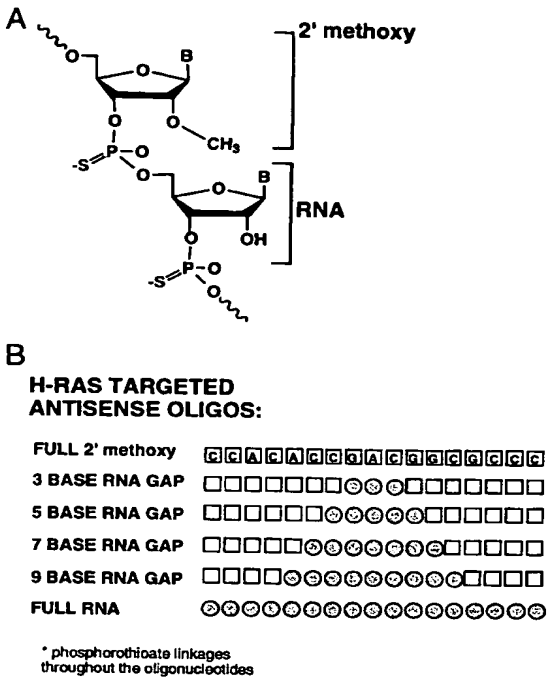

FIG. 1. **Structure of chimeric RNA gap mer oligonucleotides.** *A*, chemical structures show 2 nucleosides of a chimeric 2'-methoxy-ribonucleotide oligonucleotide molecule, with a phosphorothioate linkage between the nucleotides. *B*, schematic shows the design and composition of oligonucleotides used in this study. *Open squares* represent 2'-methoxy-modified nucleotides, *filled circles* represent ribonucleotides. Phosphorothioate linkages are present throughout all the oligonucleotides shown.

with RNA gap mer oligonucleotides containing 5, 7, and 9 ribonucleotides in the gap as well as a full phosphorothioate oligoribonucleotide molecule displayed dose-dependent reductions in Ha-Ras steady state mRNA levels (Fig. 2*B*, *top four panels*, respectively). T24 cells treated with a control 9-base RNA gap mer oligonucleotide that contained four mismatched bases in its sequence did not show dose-dependent reduction in Ha-Ras mRNA suggesting that hybridization to the target RNA was essential for activity (Fig. 2*B*, *bottom panel*). The ability of the RNA gap mer oligonucleotides to reduce Ha-Ras mRNA was dependent on the number of ribonucleotides incorporated into the RNA gap and thus the size of the RNA:RNA duplex formed in cells. The fact that the RNA gap mer oligonucleotide containing three ribonucleotides in the gap was unable to induce reduction in target mRNA suggests that the activity involved requires an RNA:RNA duplex region of at least four ribonucleotides for cleavage of the target. T24 cells treated with 600 nM of the various RNA gap mer oligonucleotides demonstrated a reduction in Ha-Ras mRNA levels of 51 ± 8% for the 5 RNA gap mer, 49 ± 4% for the 7 RNA gap mer, 77 ± 1% for the 9-base RNA gap mer, and 38 ± 5% for the full oligoribonucleotide, respectively, when compared with nontreated controls. The full phosphorothioate oligoribonucleotide molecule was slightly less active than the RNA gap mer oligonucleotides. This is probably due to the relative decrease in stability of the full oligoribonucleotide in cells resulting from inactivation by single stranded ribonucleases, as phosphorothioate 2'-methoxy modified oligonucleotides are considerably more stable than phosphorothioate oligoribonucleotides (25). This suggests that for therapeutic purposes RNA gap mer phosphorothioate oligonucleotides protected by 2'-methoxy wings (or other even more stable 2' modifications) would be more potent molecules. These

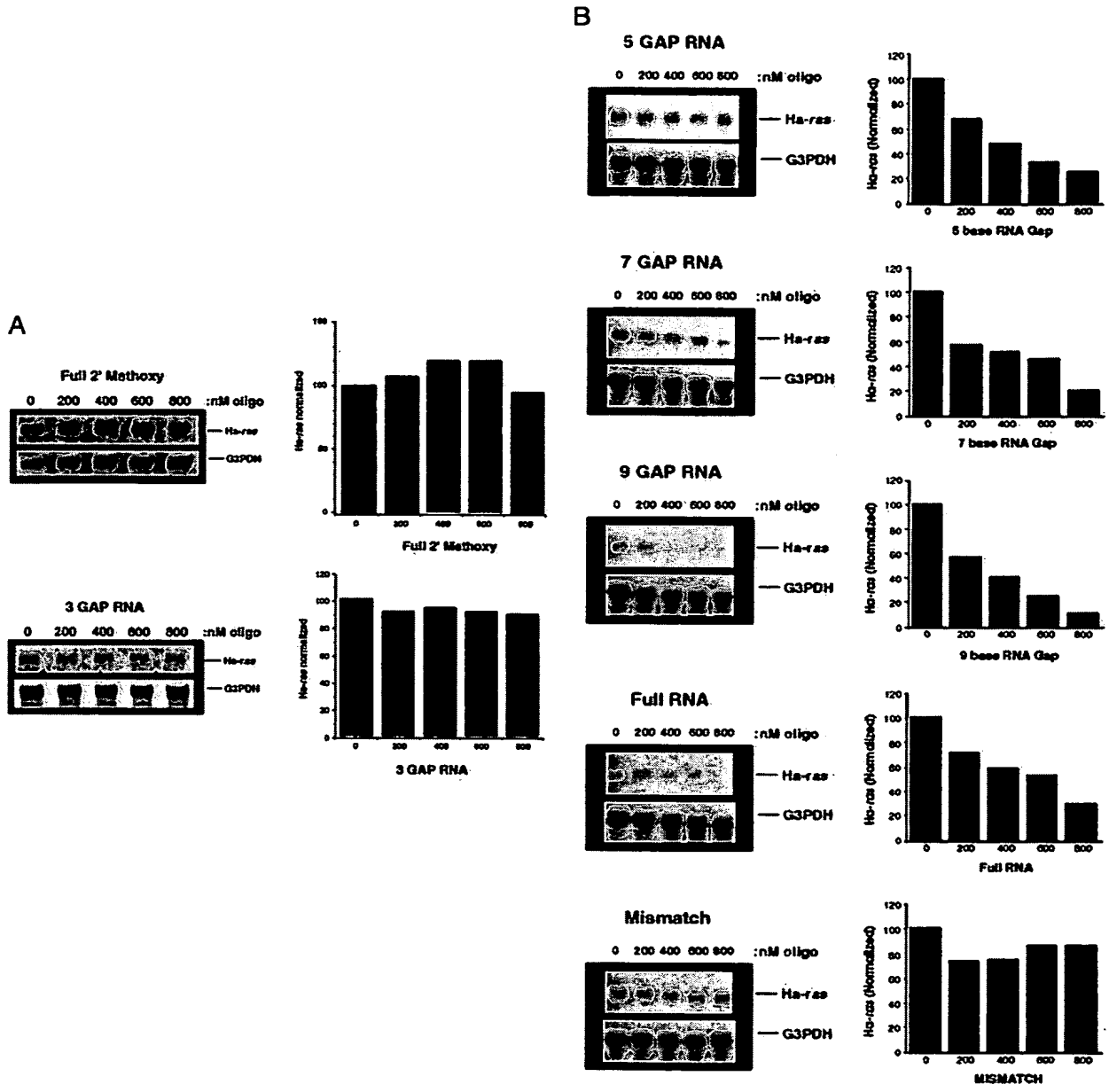

FIG. 2. **Ha-Ras mRNA levels in cells treated with full 2'-methoxy or chimeric RNA gap mer oligonucleotides.** *A*, Northern blot analyses for Ha-Ras mRNA levels in T24 cells treated with the indicated doses of full 2'-methoxy oligonucleotide (*top panel*) or 3-gap oligoribonucleotide (*bottom panel*) for 24 h. The *upper band* is the signal for Ha-Ras. This signal was normalized to that obtained for glyceraldehyde-2-phosphate dehydrogenase (*G3PDH*) (*lower band*), and relative Ha-Ras levels were determined and are presented graphically (*right panel*). Neither oligonucleotide treatment reduced Ha-Ras mRNA levels. *B*, Northern blot analyses of T24 cell treated as in *A*, except with chimeric RNA gap mer oligonucleotides containing either a 5, 7, or 9 ribonucleotide gap or a full ribonucleotide molecule (*top four panels*, respectively). Cells were also treated with a control oligoribonucleotide that contains nine ribose nucleosides with four mismatched bases to the Ha-Ras mRNA sequence (*bottom panel*). Ha-Ras signals were normalized to that of G3PDH, and relative Ha-Ras levels are shown (*right panel*).

experiments demonstrate that an endoribonuclease activity in T24 human bladder carcinoma cells recognizes the internal oligoribonucleotide:RNA portion of a chimeric duplex and reduces target mRNA levels.

An Activity Present in Human Cellular Extracts Induces Cleavage of RNA Gap Mer Oligonucleotide:RNA Duplex within the Internal RNA:RNA Portion in Vitro—To further characterize the dsRNA cleavage activity in T24 cells, we prepared T24 cellular extracts and tested these for the ability to cleave a 17-base pair duplex consisting of the 9-base RNA gap mer oligonucleotide annealed to its complementary ^{32}P-end-labeled oligoribonucleotide. The ^{32}P- labeled duplex was incubated with 20 μg of cytosolic extract at 37 °C for the indicated times (Fig. 3*A*), followed by phenol chloroform extraction, ethanol precipitation, and separation of the products on a denaturing gel. This duplex was a substrate for digestion by an activity present in T24 extracts as can be seen by the loss of full-length end-labeled RNA and the appearance of lower molecular weight digestion products (indicated by *arrows*, Fig. 3*A*). In addition, the activity responsible for the cleavage of the duplex

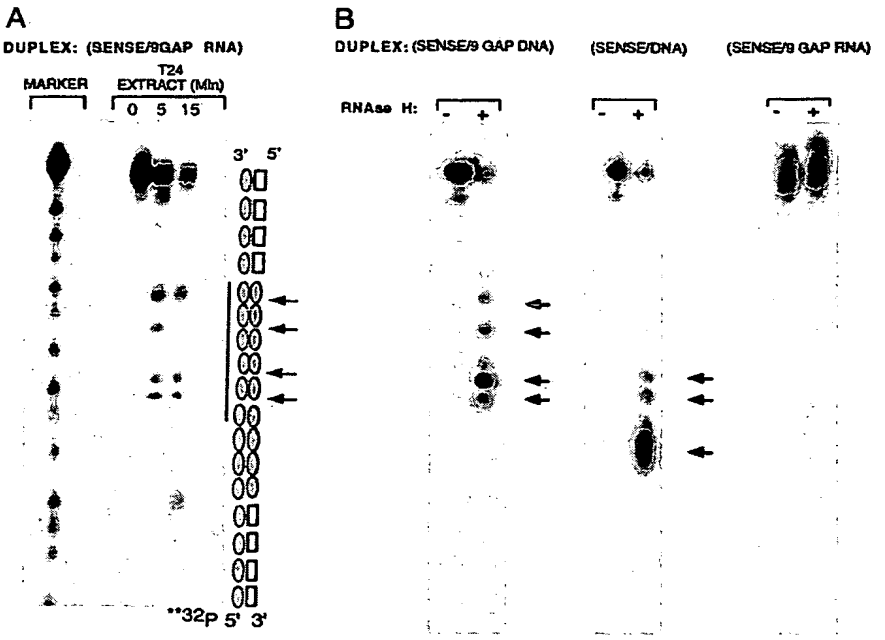

FIG. 3. **Effect of T24 cytosolic extracts and RNase H on duplexes *in vitro*.** *A*, a 17-base pair duplex consisting of the Ha-Ras targeted 9-base RNA gap mer oligonucleotide annealed to a ^{32}P-labeled RNA complement was incubated with 20 μg of a T24 cytosolic protein fraction for the indicated times at 37 °C, the reaction was stopped, and products were resolved on a denaturing polyacrylamide gel. Digestion products (*arrows*) indicate that cleavage of the duplex is restricted to the RNA:RNA region (see schematic of duplex, *far right*). *B*, the same 9-base RNA gap mer oligonucleotide:RNA duplex as in *A* was incubated with or without *E. coli* RNase H (−, +). The lack of digestion products indicates that this duplex is not a substrate for RNase H (*right panel*). Duplexes consisting of ^{32}P-labeled RNA annealed to either a full oligodeoxynucleotide (*middle panel*) or 9-base DNA gap mer oligonucleotide (*left panel*) are substrates for cleavage by RNase H and thus generate digestion products as expected (*arrows*).

displayed specificity for the RNA:RNA portion of the duplex molecule, as indicated by the sizes of the cleavage products it produced (see the physical map of the ^{32}P-end-labeled 9-base RNA gap mer:RNA duplex, Fig. 3*A*, *far right*). To evaluate the cellular distribution of this dsRNase activity, nuclear extracts were prepared from T24 cells and tested for the ability to digest the 9-base RNA gap mer oligonucleotide:RNA duplex. Nuclear extracts prepared from T24 cells were able to degrade the target duplex, and the activity was present in the nuclear fraction at comparable levels to that in the cytoplasmic fractions (data not shown). Cellular extracts prepared from human umbilical vein epithelial cells, human lung carcinoma (A549), and HeLa cell lines all contained an activity able to induce cleavage of the 9-base RNA gap mer:RNA target duplex *in vitro*. This activity was abolished by pretreatment of the extracts with proteinase K for 15 min at 65 °C (data not shown).

The initial RNA gap mer antisense oligonucleotides were synthesized to contain phosphorothioate linkages throughout the entire length of the molecule. As this results in increased stability to single strand nucleases, we reasoned that it would inhibit cleavage of the antisense strand by the dsRNase as well. Therefore, to determine if the activity we have described can cleave both strands in a RNA duplex molecule, we synthesized a 9-base RNA gap mer antisense oligonucleotide that contained phosphorothioate linkages in the wings between the 2′-methoxy nucleotides but had phosphodiester linkages between the nine ribonucleotides in the gap. A duplex comprised of the ^{32}P-labeled 9-base RNA gap mer phosphodiester/phosphorothioate antisense oligonucleotide described above and its complementary oligoribonucleotide was tested as a substrate for the dsRNase activity in T24 extracts. The activity was capable of cleaving the antisense strand of this duplex as well as the sense strand and the pattern of the digestion products indicated that cleavage was again restricted to the RNA:RNA phosphodiester portion of the duplex (data not shown).

An RNA Gap Mer Oligonucleotide:RNA Duplex Is Not a Substrate for RNase HI—To exclude the possibility that the cleavage seen might be due to an RNase H type activity, we tested the ability of *E. coli* RNase H to cleave a 17-base pair duplex composed of the 9-base RNA gap mer oligonucleotide and its complementary 5′-^{32}P-labeled oligoribonucleotide *in vitro*. As can be seen in Fig. 3*B* (*far right panel*), the 9-base RNA gap mer oligonucleotide:RNA duplex was not a substrate for RNase H cleavage as no lower molecular weight bands appeared when it was treated with RNase H. However, as expected both a full oligodeoxynucleotide:RNA duplex and a 9-base DNA gap mer oligonucleotide:RNA duplex were substrates for RNase HI under the same conditions, as is evident by the appearance of lower molecular species in the enzyme-treated lanes (Fig. 3*B*, *left* and *middle panels*). It is interesting to note that RNase HI cleavage of the 9-base DNA gap mer oligonucleotide:RNA duplex (Fig. 3*B*, *left panel*) and cleavage of the 9-base RNA gap mer oligonucleotide:RNA duplex by T24 cellular extracts resulted in similar digestion products (Fig. 3*A*). Both RNase HI and the activity in T24 cells displayed the same preferred cleavage sites on their respective duplexes. Cleavage was restricted to the 3′ end of the target RNA in the region opposite either the DNA or RNA gap of the respective antisense molecule. This suggests that RNase H and the dsRNase activity described here may share binding as well as mechanistic properties.

dsRNase Activity Generates 5′-Phosphate and 3′-Hydroxyl Termini—To determine the nature of the 5′ termini resulting from cleavage of the duplex *in vitro*, nonlabeled duplex was incubated with T24 cellular extracts as described previously, then reacted with T4 polynucleotide kinase and [γ-^{32}P]ATP with or without prior treatment with calf intestinal phosphatase. Phosphatase treatment of the duplex products was essential for the incorporation of the ^{32}P label during the reaction with polynucleotide kinase, indicating the presence of a phosphate group at 5′ termini of digestion products (data not shown). The 3′ termini of the cleaved duplex products were evaluated by the reaction of duplex digestion products with T4 RNA ligase and [^{32}P]pCp. T4 RNA ligase requires a free 3′-hydroxyl terminus for the ligation of [^{32}P]pCp. The ability of the duplex digestion products to incorporate [^{32}P]pCp by T4 RNA ligase indicated the presence of 3′-hydroxyl groups (data not shown).

dsRNase Activity in Rat Liver—To determine if non-human mammalian cells contain dsRNase activity, and to provide a

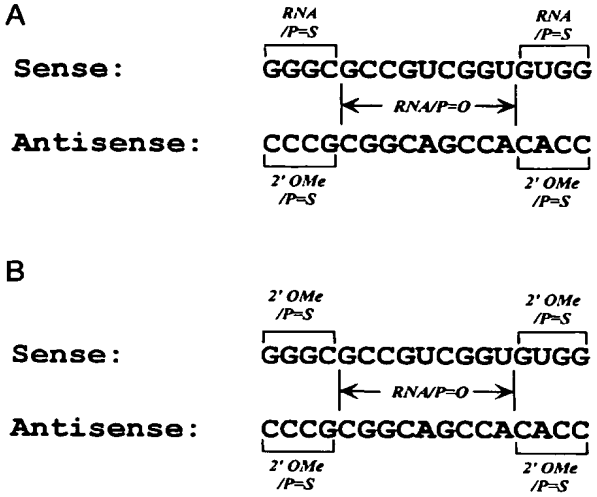

FIG. 4. **Two sets of duplex oligoribonucleotide substrates for the dsRNase activity assay in nondenaturing and denaturing acrylamide gel assays.** *P=O*, phosphodiester linkage; *P=S*, phosphorothioate linkage; *2' Ome*, 2'-methoxy nucleoside. *A*, sense strand has P=S in the wings. *B*, sense strand was 2' Ome and P=S in the wings.

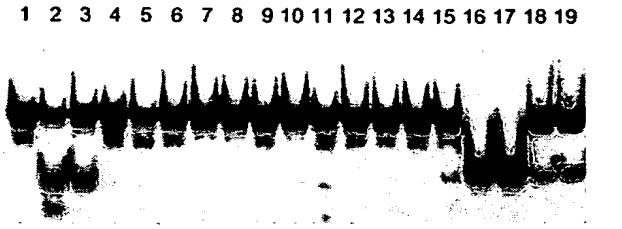

FIG. 5. **Cleavage of substrates by rat liver cytosolic and nuclear extracts.** Antisense and sense oligonucleotides were annealed and incubated with the cellular extracts and variety of RNases, then subjected to native 12% acrylamide gel, as described under "Methods and Materials." *Lane 1*, RNA duplex substrate; *lanes 2* and *3*, duplex digested with partially purified rat liver cytosolic (1 μg) or nuclear extract (0.1 μg); *lane 4*, RNase A (10^{-4} units); *lanes 5* and *6*, RNase CL3 (1 and 10^{-1} unit); *lanes 7* and *8*, partially purified calf thymus RNase H (1/5 and 1/50 unit); *lanes 9* and *10*, *E. coli* RNase H_1 (1/400 and 1/4000 unit); *lanes 11* and *12*: RNase T1 (10^{-1} and 10^{-2} unit); *lanes 13* and *14*, RNase T2 (1 and 10^{-1} unit); *lane 15*, RNase S1 (1 unit); *lanes 16* and *17*, RNase V_1 (1 and 10^{-1} unit); *lanes 18* and *19*, T24 cellular extract (20 and 40 μg).

source from which the activity might be purified, we chose rat liver. In preliminary experiments, dsRNase activity was observed in rat liver homogenates, but the homogenates also displayed higher levels of single strand RNases that confounded analysis because of cleavage of the oligoribonucleotide overhangs after cleavage by dsRNase. To solve this problem, we used two additional substrates and a nondenaturing gel assay. The "antisense" strand in both substrates contained 2'-methoxyphosphorothioate wings on either side of an nine-base ribonucleotide phosphodiester gap. The "sense" strand was either an oligoribonucleotide, with phosphodiester in the 9-base gap flanked by phosphorothioate linkages (Fig. 4*A*), or had flanks comprised of 2'-methoxy nucleosides with phosphorothioate linkages (Fig. 4*B*). Both substrates were more stable to exonuclease digestion than an oligoribonucleotide, and the substrate with phosphorothioate linkages and 2'-methoxy nucleosides in both strands was extremely stable. This was important because of the abundance of single strand RNases relative to the dsRNase activity in the liver and supported the use of nondenaturing assays, as the products of the cleavage by dsRNase remained double-stranded.

Rat liver cytosolic and nuclear extracts induced cleavage of the duplex substrate (Fig. 5, *lanes 2* and *3*). Both extracts resulted in more rapidly migrating bands on native gel electrophoretic analyses. A dsRNase, RNase V1 cleaved the substrate (*lanes 16* and *17*); T24 extracts also cleaved the substrate (*lanes 18* and *19*). Neither bacterial nor human RNase H, nor single-strand RNases cleaved the substrate (*lanes 4–15*).

Fig. 6*A* shows the elution profile of the rat liver cytosolic extract after ion-exchange chromatography. Fig. 6*B* shows that the dsRNase activity eluted in fractions 53–63 (300–450 mM NaCl). In contrast, the dsRNase activity in the nuclear extract eluted at 700–800 mM NaCl (Fig. 6, *C* and *D*). In some chromatographic separations, activities that eluted at both high and low NaCl concentrations were observed in the cytosol and the nucleus.

Fractions from the ion-exchange chromatography of rat liver cytosol were concentrated and subjected to size exclusion chromatography as described under "Materials and Methods." Fig. 7*A* shows the elution profile and Fig. 7*B* the activity profile of cytosolic dsRNase after size-exclusion chromatography. Fig. 7*C* shows a polyacrylamide gel electrophoretic analysis of the concentrated active fractions, after the ion-exchange chromatography, and the fractions from the size exclusion chromatography. The fraction with greatest dsRNase activity (*fraction 3*) had a mean molecular mass of 45–80 kDa, and two bands at approximately 50 kDa appeared to be enhanced on polyacrylamide gel analysis. Comparison of the gel analysis of fractions 3 and 4 shows that proteins of approximately 40 and 64 kDa did not co-purify with the dsRNase activity. *Lane 5* shows that a protein of approximately 55 kDa did not co-purify with the activity. Obviously, fraction 3 represents only a partially purified fraction. Table I provides a summary of the purification and recovery of dsRNase activities from nuclear and cytosolic liver extracts. Purification of the protein(s) responsible for the nuclear activity has proven more difficult and will be the subject of an additional communication.

The effects of various conditions on the dsRNase activity were evaluated using the active fractions after ion-exchange chromatography. Fig. 8 shows that dsRNase activity was apparent in a Tris or phosphate buffer at pH 7–10 (*lanes 1–15*). It was unstable in acetonitrile or methanol (*lanes 42* and *43*) and was inhibited by NaCl; dsRNase activity was inhibited by 30% at 10 mM, >60% at 100 mM, and 100% at 300 mM NaCl (*lanes 36–40*). Heating for 5 min at 60 °C inactivated the enzyme (*lanes 21–23*), and the activity had a temperature optimum of 37–42 °C (*lanes 27–29*). At 25 °C, the activity was approximately 50% of that observed at 37 °C (*lane 30*). The activity was inhibited by EDTA (*lanes 31–35*), required Mg^{2+} and was stable to multiple freeze/thaws (*lanes 24–26*). It also was ablated by treatment with proteinase K (data not shown).

Cleavage Characteristics—To characterize the site of cleavage in more detail, it was necessary to minimize single strand cleavage that occurred after endonuclease cleavage and during handling, particularly after denaturing of the duplex. Consequently, we used the most stable duplex substrate in which both strands of the duplex contained flanking regions comprised of 2'-methoxy nucleosides and phosphorothioate linkages.

Fig. 9*A* displays the results from native gel analyses. *Lane 1* shows the position at which the ^{32}P-labeled sense strand migrated in the native gel. *Lane 2* shows that the "sense" single strand was not digested by dsRNA-specific ribonuclease V1. *Lanes 3* and *4* show the degradation of RNA duplexed with antisense RNA gap mer resulting from high and low concentrations of V1 RNase. *Lanes 5* and *6* show that crude nuclear extract degraded the duplex in a Mg^{2+}-dependent fashion.

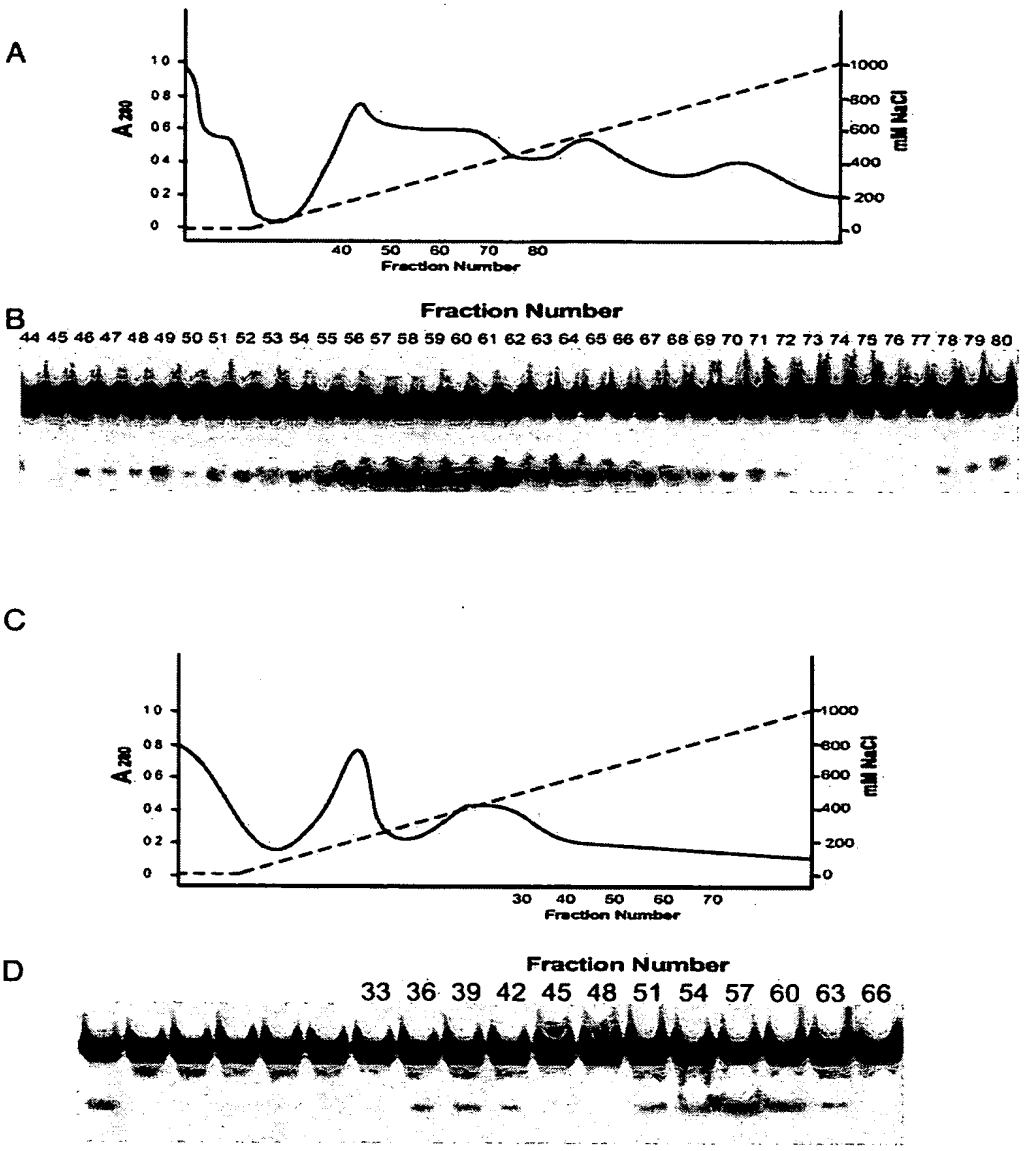

FIG. 6. **Ion-exchange chromatograph of dsRNase activity from rat liver cytosolic (*A* and *B*) and nuclear (*C* and *D*) extracts.** After NH_4Cl precipitation and dialysis with buffer A, the extracts were loaded onto a 100-ml Hi-Trap SP ion-exchange column and eluted by a 0–1 M NaCl increase gradient (----). *A* and *C*, elution profile; *B* and *D*, dsRNase activity of the fraction (1–2 μl) was determined as described uder "Materials and Methods."

Lane 7 shows that crude cytosolic extract also induced cleavage of the substrate. Ion-exchange purified cytosolic extract cleaved the substrate in a Mg^{2+}-dependent fashion as well (*lanes 8* and *9*). Active fractions alter size exclusion chromatography also cleaved the substrate in a Mg^{2+}-dependent fashion (*lanes 10* and *11*).

Fig. 9*B* shows the denaturing gel analysis of the degradation products. *Lane 1* shows the products of a limit digest of the single-strand sense oligonucleotide. The position of the degradate is consistent with it being the 2′-methoxyphosphorothioate-flanking region (wing). RNase V1 digestion of the single-strand substrate resulted in little degradation (*lane 2*). RNase V1 digestion of the duplex resulted in degradates reflecting cleavage at several sites within the dsRNA gap (*lane 3* and *4*). In *lanes 4–14*, the band at the *top* of the gel demonstrates that, even after denaturation, some of the duplex remained annealed, reflecting the very high affinity of duplexes comprised of 2′-methoxy nucleosides. *Lanes 6–9* show that both the nuclear and cytosolic enzymes cleaved the duplex substrate at several sites within the oligoribonucleotide gap and that the sites of degradation were different from those of V1 nuclease.

DISCUSSION

By the rational design of chemically modified antisense oligonucleotides that contain oligoribonucleotide stretches of varying length, we have identified an activity in cells and rat liver that requires the formation of a dsRNA region to degrade target RNA. This activity is present at comparable levels in both the nuclear and cytoplasmic fractions of T24 human bladder carcinoma cells. We have found that this activity produces 5′-phosphate and 3′-hydroxyl termini after cleavage of its RNA substrate. The generation of 5′-phosphate and 3′-hydroxyl ter-

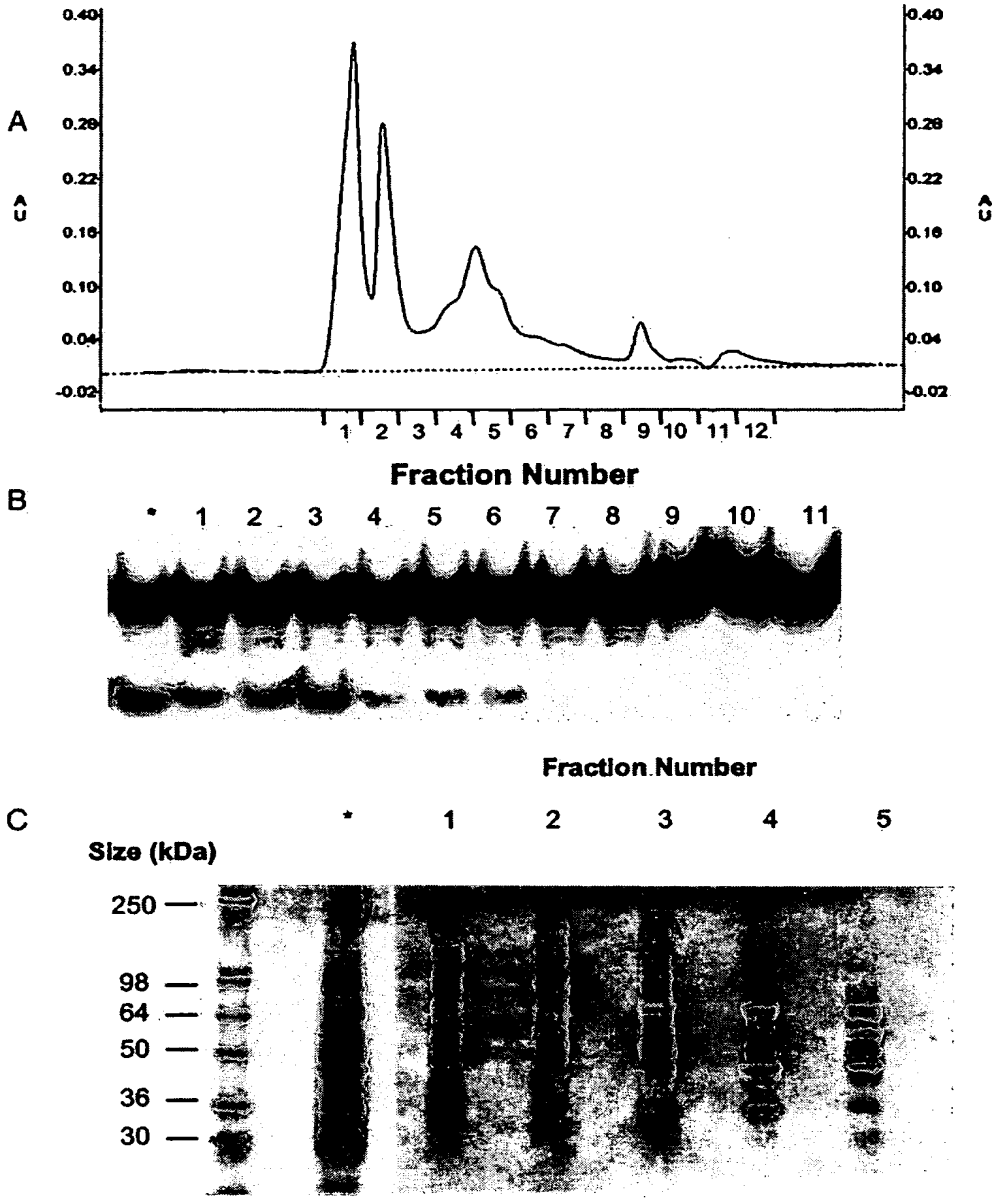

FIG. 7. **Gel filtration of dsRNase activity from rat liver cytosolic extracts.** Extract after ion-exchange was concentrated and loaded onto a TSK3000 gel filtration column. *A*, elution profile; *B*, dsRNase activity for the fractions (1 μl); and *C*, SDS-polyacrylamide gel electrophoresis with Coomassie Blue stain (6 μg of protein from each fraction). * = sample after ion-exchange chromatography only.

TABLE I
Partial purification of dsRNase from rat liver extracts
Fractions from rat liver nuclei and cytosol were prepared and tested as described under "Materials and Methods."

Fraction	Protein	Total activity	Specific activity	Purification factor	Recovery
	mg	*unit*[a]	*unit/mg*		%
Cytosolic extract	30,000	1,020,000	34	1	100
Ion-exchange pool	991	459,000	463	14	56
Gel filtration pool	18.4	100,980	5,600	165	22
Nuclear extract	5,000	205,000	41	1	100
Ion-exchange pool	11.2	77,900	6970	170	38

[a] Unit is the amount of enzyme required to digest 10 fmol of dsRNA duplex in 15 min at 37 °C in the condition described under "Materials and Methods."

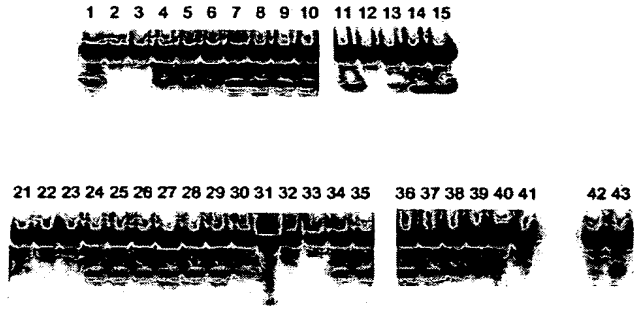

FIG. 8. **Effect of various conditions on dsRNase activity.** 1 μg partially purified rat liver cytosolic extract was incubated with duplex substrate as described under "Materials and Methods." *Lanes 1* and *11*, 20 mM Tris buffer (pH 7.5); *lanes 2–6*, 20 mM sodium acetate buffer (pH 4.5, 5.5, 6.0, 7.0, and 8.0); *lanes 7–10*, 20 mM Tris buffer (pH 7.0, 8.0, 9.0, and 10.0); *lanes 12–15*, 20 mM sodium phosphate buffer (pH 5.0, 6.0, 7.0, and 8.0); *lanes 21–23*, 60, 80, and 100 °C, incubation of extract for 5 min prior to digestion of duplex substrate; *lanes 24–26*, repeat cycles of freezing and thawing 10, 3, and 0 times; *lanes 27–30*, digestion reaction incubated at 50, 42, 37 and 22 °C; *lanes 31–35*, reaction buffer with final EDTA concentration of 50, 20, 10, 5 and 0 mM; *lanes 36–40*, reaction buffer with final NaCl concentration of 30, 100, 300, 500, and 1000 mM; *lane 41*, substrate only; *lanes 42* and *43*, extract pretreated with organic solvent (60% methanol and acetonitrile).

mini is a common feature of several other nucleases that recognize double strand nucleic acid molecules, including RNase HI (26), the enzyme that cleaves the RNA component of a DNA:RNA duplex, and *E. coli* RNase III, which catalyzes the hydrolysis of high molecular weight dsRNA and mediates degradation of sense-antisense duplexes (27). The fact that both the oligoribonucleotide portion of the 9-base RNA gap mer strand in the 9-base RNA gap mer oligonucleotide:RNA duplex as well as the RNA strand were cleaved by this activity demonstrates that the enzyme(s) can specifically recognize and cleave both strands of an RNA:RNA type duplex. The presence of phosphorothioate linkages in the antisense molecule should prevent cleavage of this strand when administered to cells and therefore enhance the potential of such compounds to have therapeutic utility. Interestingly, cleavage of both strands does not seem to be required, in that target mRNA was greatly reduced even though phosphorothioate RNA gap mer antisense oligoribonucleotides were used.

The partial purification of the activity from liver nuclear and cytosolic extracts suggests that the activity is present in both subcellular compartments in rat liver cells as well as human cell lines. The nuclear enzyme eluted from the ion-exchange column at higher NaCl concentrations than did the cytosolic enzymes. However, both require Mg^{2+} and cleave at several sites within the oligoribonucleotide gap. Both require a duplex substrate. This may suggest that there are different types of proteins with dsRNase activity in nuclei and cytosol, but much more work is required before conclusions can be drawn. Additionally, as the nuclear activity eluted at a different NaCl concentration than did the cytosolic, it seems likely that the nuclear activity did not contribute to the cytosolic activity that eluted at lower NaCl concentrations. However, in several preparations, there was evidence of small amounts of activity that eluted at 700–800 mM NaCl in the cytosol, and this could have been due to nuclear contamination. Again, only additional work will definitively determine the cellular localization of the activities.

Many components of mRNA degradation systems have been conserved between pro- and eukaryotes (28, 29). Here we show that like some prokaryotic organisms, in which RNase III carries out the degradation of sense-antisense hybrids to regulate the expression of some genes, human cells have conserved an activity capable of performing a similar role. For some time the dsRNA adenosine deaminase enzyme was suggested to target RNA hybrids for degradation by some unknown mechanism (30). However, more recently it has been demonstrated that deaminated transcripts are usually at least as stable as unmodified RNA (31). This enzyme efficiently modifies duplexes containing 100 base pairs or more and would therefore not be a factor in our system where dsRNA regions ranged from 3 to a maximum of 17 base pairs. In addition, Ha-Ras mRNA does not contain any adenosine residues in the region targeted by our antisense oligonucleotides. The identification of a human dsRNase activity may help us understand how human cells use endogenously expressed antisense transcripts to modulate gene expression. It also has important implications for antisense therapeutics.

The activities reported in this study appear to be novel. The properties of the proteins responsible for cleavage of the substrates are clearly different from other enzymes reported. For example, the dsRNase induced by interferon has a different molecular weight, salt and divalent ion requirements, and is secreted (18). We have not observed dsRNase H activity in cell supernatants.

The vast majority of antisense oligonucleotides used experimentally or currently being tested in the clinic are modified oligodeoxynucleotides (1, 7). It has been demonstrated that the heteroduplex formed between such oligodeoxynucleotide antisense compounds and their target RNA is recognized by the intracellular nuclease RNase H that cleaves only the RNA strand of this duplex. Although RNase H-mediated degradation of target RNA has proven a useful mechanism, it has limitations. One is the fact that the oligonucleotide must be "DNA-like," and such oligonucleotides have inherently a lower affinity for their target RNA. Strategies designed to circumvent this lower affinity include the design of gap mer oligonucleotides that are comprised of a stretch of high affinity chemically modified oligonucleotides on the 5′ and 3′ ends (the wings) with a stretch of deoxynucleotides in the center (the gap) (7, 23). DNA gap mer oligonucleotides have significantly higher affinities for their target. However, depending on the size of the DNA gap, RNase H activity may also be compromised (7, 23). The cellular localization and tissue distribution of RNase H activity are also concerns for antisense therapy. RNase H activity is primarily localized to the nucleus (32), although it has been detected at lower levels in the cytoplasm. RNase H activity is also variable from cell line to cell line and between tissues (8), thus a given disease state may not be a good candidate for antisense therapy, simply because the target tissue has insufficient RNase H activity. Finally, and perhaps most importantly, the majority of sites within RNA targets that have been studied are not sensitive to RNase H-induced cleavage (8). It is clear then that alternative terminating mechanisms to RNase H activation are required for widespread application of antisense therapeutics.

The activity described in this work is attractive as an alternative terminating mechanism to RNase H for antisense therapeutics. The activity relies upon "RNA-like" oligonucleotides that have higher affinity for their target and thus should have higher potency than "DNA-like" oligonucleotides. The presence of the activity in both the cytoplasm and the nucleus suggests that it might be used to inhibit many RNA processing events from nuclear pre-mRNA splicing and transport to the degradation of mature transcripts in the cytoplasm. As we have examined the dsRNase activity induced only by the RNA gap mer oligonucleotides targeted to codon 12 of Ha-Ras, it is difficult to estimate the relative abundance of this dsRNase activity or

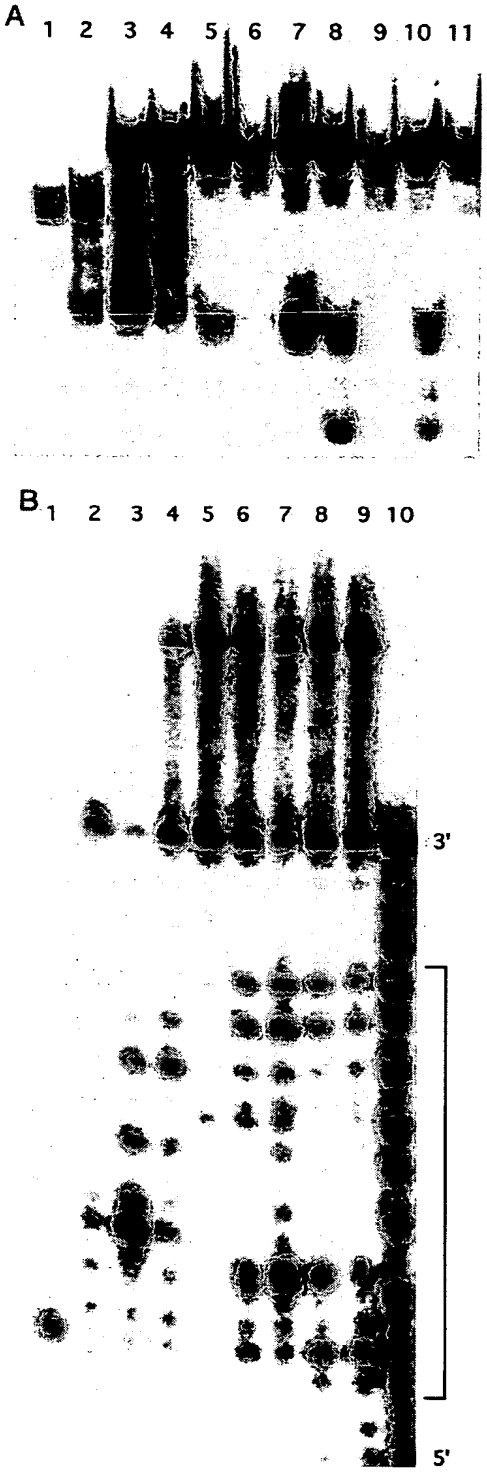

FIG. 9. **Analysis of dsRNA oligonucleotide digestion products by native polyacrylamide gel electrophoresis.** *A*, antisense and sense oligonucleotides were preannealed and incubated with the cellular extracts as described under "Materials and Methods." Polyacrylamide gel analysis of the digestion products was performed as described under "Materials and Methods." Sense strand RNA alone (*lane 1*) and digested with RNase V1 (*lane 2*) are shown. RNase V1 digestion of single strand sense oligonucleotide was performed in 10 μl containing 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 10^4 cpm RNA, and 0.5 unit of RNase V1. RNase V_1 digestion of dsRNA was prepared as above with the exception that 10^4 cpm of sense oligonucleotide was preannealed with 10 nM antisense oligonucleotide prior to digestion with 2×10^{-2} units of RNase V1 (*lane 3*) and 2×10^{-3} units of RNase V1 (*lane 4*). RNase reactions were incubated at 37 °C for 5 min. The digestion patterns for the dsRNA oligonucleotide incubated with the various cellular extracts are as follows: unpurified nuclear extract incubated for 240 min (*lane 5*); unpurified nuclear extract incubated for 240 min in the absence of $MgCl_2$ (*lane 6*); unpurified cytosolic extract incubated for 240 min (*lane 7*); ion-exchange purified cytosolic extract incubated for 240 min (*lane 8*); ion-exchange purified cytosolic extract incubated for 240 min in the absence of $MgCl_2$ (*lane 9*); ion-exchange and gel filtration-purified cytosolic extract incubated for 240 min (*lane 10*); ion-exchange and gel filtration-purified cytosolic extract incubated for 240 min in the absence of $MgCl_2$ (*lane 11*). *B*, analysis of dsRNA oligonucleotide digestion products by denaturing polyacrylamide gel electrophoresis. The *bracketed region* indicates the position of the RNA gap. RNase A and V1 digestions of single strand sense oligonucleotide were performed in 10 μl containing 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 10^4 cpm of ^{32}P-labeled RNA and 5×10^{-4} units of RNase A (*lane 1*) or 2×10^{-2} units of RNase V_1 (*lane 2*). RNase V_1 digestion of dsRNA was performed as described above at 2×10^2 units (*lane 3*) or 2×10^3 units (*lane 4*). The digestion patterns for the dsRNA oligonucleotide incubated with the various cellular extracts are as follows: unpurified nuclear extract incubated for 0 min (*lane 5*); unpurified nuclear extract incubated for 240 min (*lane 6*); unpurified cytosolic extract incubated for 240 min (*lane 7*); ion-exchange-purified cytosolic extract incubated for 240 min (*lane 8*); ion-exchange and gel filtration-purified cytosolic extract incubated for 240 min (*lane 9*). The base hydrolysis ladder was prepared by incubation of the 10^4 cpm RNA at 90 °C for 5 min in 10 μl containing 100 mM sodium carbonate, pH 9.0 (*lane 10*).

potential potency of these RNA gap mer compounds for other sites compared with RNase H active oligonucleotides. The target site in codon 12 of Ha-Ras is one of the most RNase H-sensitive sites we have identified. A phosphorothioate oligodeoxynucleotide to that site typically displays an IC_{50} of approximately 50 nM in T24 cells (22). The IC_{50} for the 9-base RNA gap mer oligonucleotide was approximately 200 nM, suggesting that this activity is capable of degrading this site nearly as well as RNase H.

The selective inhibition of mutated genes such as the *ras* oncogene necessitates antisense hybridization in the coding region of the mRNA. This requires either a high affinity interaction between oligonucleotide and mRNA to prevent displacement of the oligonucleotide by the polysome or rapid degradation of the target mRNA. RNA gap mer oligonucleotides, being inherently higher in affinity than oligodeoxynucleotides and being able to take advantage of a cellular dsRNase activity, may satisfy both these criteria. Identification of sites that are differentially sensitive to RNase H and to dsRNase activities will increase the number of potential target sites on a given mRNA for antisense oligonucleotides.

It is clear that an activity capable of degrading dsRNA must be carefully regulated, since dsRNA and stem loop structures abound in all cells and uncontrolled cleavage of such substrates would surely be toxic. Mechanisms of regulation may include direct inhibitors and activators, cellular compartmentalization, and regulation by cellular signal transduction pathways. One such pathway that could potentially be involved is the dsRNA-activated protein kinase pathway (33). The kinase p68, which is induced by dsRNA or interferon, phosphorylates the eukaryotic translation initiation factor 2, which results in translational inhibition.

Further purification, characterization, and cloning of the dsRNase activity presented here will be required to increase understanding of its cellular function and regulation. Clearly, the enzyme(s) may play important roles in the intermediary metabolism of RNA and may be involved in the degradation of RNA species targeted by natural antisense transcripts. Drugs

designed to take advantage of this mechanism may help increase the scope of antisense-based therapeutics.

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EXHIBIT G

Declaration of Dr. Arthur Riggs

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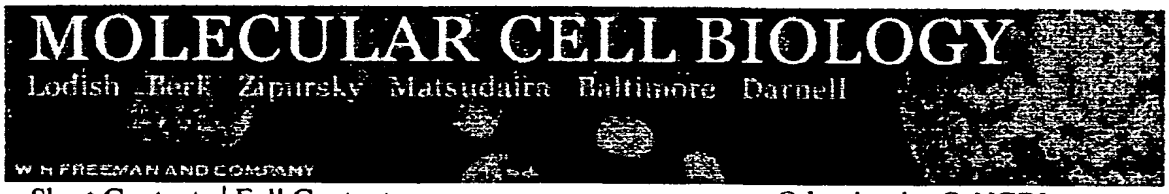

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Molecular Cell Biology ➔ **11. RNA Processing, Nuclear Transport, and Post-Transcriptional Control**

11.2. Processing of Eukaryotic mRNA

As discussed in Chapter 4, the initial primary transcript synthesized by RNA polymerase II undergoes several processing steps before a functional mRNA is produced. In this section, we take a closer look at how eukaryotic cells carry out mRNA processing, which includes three major processes: **5′ capping, 3′ cleavage/polyadenylation,** and RNA splicing (Figure 11-7). Processing occurs in the nucleus, and the functional mRNA produced is transported to the cytoplasm by mechanisms discussed later.

The 5′-Cap Is Added to Nascent RNAs Shortly after Initiation by RNA Polymerase II

After nascent RNA molecules produced by RNA polymerase II reach a length of 25 – 30 nucleotides, *7-methylguanosine* is added to their 5′ end. This initial step in RNA processing is catalyzed by a dimeric capping enzyme, which associates with the phosphorylated carboxyl-terminal tail domain (CTD) of RNA polymerase II. Recall that the CTD becomes phosphorylated during transcription initiation (see Figure 10-50). Because the capping enzyme does not associate with polymerase I or III, capping is specific for transcripts produced by RNA polymerase II.

One subunit of the capping enzyme removes the γ-phosphate from the 5′ end of the nascent RNA emerging from the surface of a RNA polymerase II (Figure 11-8). The other subunit transfers the GMP moiety from GTP to the 5′-diphosphate of the nascent transcript, creating the guanosine 5′-5′-triphosphate structure. In the final steps, separate enzymes transfer methyl groups from *S*-adenosylmethionine to the N_7 position of the guanine and the 2′ oxygens of riboses at the 5′ end of the nascent RNA. ↑ TOP

Pre-mRNAs Are Associated with hnRNP Proteins Containing Conserved RNA-Binding Domains

Nascent RNA transcripts from protein-coding genes and mRNA processing intermediates, collectively referred to as pre-mRNA, do not exist as free RNA molecules in the nuclei of eukaryotic cells. From the time nascent transcripts first emerge from RNA polymerase II until mature mRNAs are transported into the cytoplasm, the RNA molecules are associated with an abundant set of nuclear proteins, as numerous in growing eukaryotic cells as histones. These proteins are the major protein

components of heterogeneous ribonucleoprotein particles (hnRNPs), which contain *heterogeneous nuclear RNA* (hnRNA), a collective term referring to pre-mRNA and other nuclear RNAs of various sizes. The proteins in these ribonucleoprotein particles can be dramatically visualized with fluorescentlabeled monoclonal antibodies (Figure 11-9).

To identify **hnRNP proteins,** researchers exposed cells to high-dose UV irradiation, which causes covalent cross-links to form between RNA bases and closely associated proteins. Chromatography of nuclear extracts from treated cells on an oligo-dT cellulose column, which binds RNAs with a poly(A) tail, was used to recover proteins that had become cross-linked to nuclear mRNA in living cells (i.e., hnRNP proteins). Subsequent treatment of cell extracts from unirradiated human cells with monoclonal antibodies specific for the major hnRNP proteins identified by this cross-linking technique revealed a complex set of abundant hnRNP proteins ranging in size from 34 to 120 kDa. Characterization of the mRNAs encoding these proteins has shown that some of them (e.g., A2 and B1) are related proteins derived by alternative splicing of exons from the same transcription unit.

Binding studies with purified hnRNP proteins suggest that different hnRNP proteins associate with different regions of a newly made pre-mRNA molecule as determined by the sequence of the RNA. For example, the hnRNP proteins A1, C, and D bind preferentially to the pyrimidine-rich sequences at the 3′ ends of introns, discussed in a later section. Like transcription factors, most hnRNP proteins have a modular structure. They contain one or more RNA-binding domains and at least one other domain that is thought to interact with other proteins. Several different RNA-binding motifs have been identified by constructing deletions of hnRNP proteins and testing their ability to bind RNA. Although some RNA-binding proteins contain domains with the zinc-finger motif common in DNA-binding proteins (see Figure 10-41), this motif has not yet been described in any hnRNP proteins.

The *RNP motif,* also called the RNA-binding domain (RBD), is the most common RNA-binding domain in hnRNP proteins. This ≈80-residue motif, which occurs in many other RNA-binding proteins, contains two highly conserved regions (RNP1 and RNP2) that allow the motif to be recognized in newly sequenced proteins. X-ray crystallographic analysis has shown that the RNP motif consists of a four-stranded β sheet flanked on one side by two α helices. The conserved RNP1 and RNP2 sequences lie side by side on the two central β strands, and their side chains make multiple contacts with a single-stranded region of RNA. The single-stranded RNA loop lies across the surface of the β sheet and fits into a groove between the protein loop connecting strands β_2 and β_3 and the C-terminal region (Figure 11-10).

The *RGG box,* another RNA-binding motif found in hnRNP proteins, contains five Arg-Gly-Gly (RGG) repeats with several interspersed aromatic amino acids. Although the structure of this motif has not yet been

Overview of mRNA processing in...
Figure 11-8. Capping of the 5′ end...
Figure 11-9. Visualization of hnRNP protein associated...
Figure 11-10. Structure of complex between an...
Figure 11-11. Hybridization of RNA molecules in...
Figure 11-12. Model for cleavage and polyadenylation...
Figure 11-13. Demonstration that introns are spliced...
Figure 11-14. Consensus sequences around 5′ and...
Figure 11-15. Analysis of RNA products formed...
Figure 11-16. Splicing of exons in pre-mRNA...
Figure 11-17. Diagram of interactions between pre-mRNA,...
Figure 11-18. Electron micrograph of a spliceosome.
Figure 11-19. The

determined, its arginine-rich nature is similar to the RNA-binding domains of the λ-phage N and HIV Tat proteins.

The 45-residue *KH motif* is found in the hnRNP K protein and several other RNA-binding proteins; commonly two or more copies of the KH motif are interspersed with RGG repeats. The three-dimensional structure of a representative KH motif, determined by NMR methods (Section 3.5), is similar to that of the RNP motif but smaller, consisting of a three-stranded β sheet supported from one side by a single α helix. It is not yet clear how this motif binds RNA. Mutations in the fragile-X gene *(FMR1),* which encodes a protein containing the KH motif, are associated with the most common form of heritable mental retardation. Although the molecular function of the Fmr1 protein is unknown, it presumably involves RNA binding. TOP

hnRNP Proteins May Assist in Processing and Transport of mRNAs

The association of pre-mRNAs with hnRNP proteins may prevent formation of short secondary structures dependent on base-pairing of complementary regions, thereby making the pre-mRNAs accessible for interaction with other macromolecules (Figure 11-11). Moreover, pre-mRNAs associated with hnRNP proteins present a more uniform substrate for further processing steps than would free, unbound pre-mRNAs each type of which forms a unique secondary structure dependent on its specific sequence.

The diversity of hnRNP proteins suggests that they probably have other functions as well. For example, various hnRNP proteins may interact with the RNA sequences that specify RNA splicing or cleavage/polyadenylation and contribute to the structure recognized by RNA-processing factors. Finally, cell-fusion experiments have shown that some hnRNP proteins remain localized in the nucleus, whereas others cycle in and out of the cytoplasm, suggesting that they function in the transport of mRNA (see later section). TOP

Pre-mRNAs Are Cleaved at Specific 3′ Sites and Rapidly Polyadenylated

In animal cells, all mRNAs, except histone mRNAs, have a 3′ poly(A) tail. Early studies of pulse-labeled adenovirus and SV40 RNA demonstrated that the viral primary transcripts extend beyond the poly(A) site in the viral mRNAs. These results suggested that A residues are added to a 3′ hydroxyl generated by endonucleolytic cleavage, but the predicted downstream RNA fragments are degraded so rapidly in vivo that they cannot be detected. However, this cleavage mechanism was firmly established by detection of both predicted cleavage products in in vitro processing reactions performed with extracts of HeLa-cell nuclei.

Early sequencing of cDNA clones from animal cells showed that nearly all mRNAs contain the sequence AAUAAA 10 – 35 nucleotides upstream from the poly(A) tail. Polyadenylation of RNA transcripts from transfected

spliceosomal splicing cycle.
Figure 11-20. Schematic diagrams comparing the secondary...
Figure 11-21. Localization of polyadenylated RNA and...
Figure 11-22. Transmission electron micrograph showing the...

genes is virtually eliminated when template DNA encoding the AAUAAA sequence is mutated to any other sequence except one encoding AUUAAA. The unprocessed RNA transcripts produced from such mutant templates do not accumulate in nuclei, but are rapidly degraded. Further mutagenesis of sequences within a few hundred bases of poly(A) sites revealed that a second signal downstream from the cleavage site is required for efficient **cleavage and polyadenylation** of most pre-mRNAs in animal cells. This downstream poly(A) signal is not a specific sequence but rather a GU-rich or simply a U-rich region within ≈50 nucleotides of the cleavage site.

Identification and purification of the proteins required for cleavage and polyadenylation of pre-mRNA has led to the model shown in Figure 11-12. According to this model, a 360-kDa *cleavage and polyadenylation specificity factor* (CPSF), composed of four different polypeptides, first forms an unstable complex with the upstream AU-rich poly(A) signal. Then at least three additional proteins — a 200-kDa heterotrimer called *cleavage stimulatory factor* (CStF), a 150-kDa heterotrimer called *cleavage factor I* (CFI), and a second cleavage factor (CFII), as-yet poorly characterized — bind to the CPSF-RNA complex. Interaction between CStF and the GU- or U-rich downstream poly(A) signal stabilizes the multiprotein complex. Finally, a *poly(A) polymerase* (PAP) binds to the complex before cleavage can occur. This requirement for PAP binding links cleavage and polyadenylation, so that the free 3′ ends generated are rapidly polyadenylated. Assembly of this large, multiprotein cleavage-polyadenylation complex around the AU-rich poly(A) signal in a pre-mRNA is analogous in many ways to formation of the transcription-initiation complex at the AT-rich TATA box of a template DNA molecule (see Figure 10-50). In both cases, multiprotein complexes assemble cooperatively through a network of specific protein – nucleic acid and protein-protein interactions.

Following cleavage at the poly(A) site, polyadenylation proceeds in two phases. Addition of the first 12 or so A residues occurs slowly, followed by rapid addition of up to 200 – 250 more A residues. The rapid phase requires the binding of multiple copies of a poly(A)-binding protein containing the RNP motif. This protein is designated *PABII* to distinguish it from the poly(A)-binding protein that binds to the poly(A) tail of cytoplasmic mRNAs. PABII binds to the short A tail initially added by PAP, stimulating polymerization of additional A residues by PAP (see Figure 11-12). PABII is also responsible for signaling poly(A) polymerase to terminate polymerization when the poly(A) tail reaches a length of 200 – 250 residues, although the mechanism for measuring this length is not yet understood. ↑ TOP

Splicing Occurs at Short, Conserved Sequences in Pre-mRNAs via Two Transesterification Reactions

During the final step in formation of a mature, functional mRNA, the introns are removed and exons are spliced together (see Figure 11-7). The discovery that introns are removed during splicing came from electron

MOLECULAR CELL BIOLOGY
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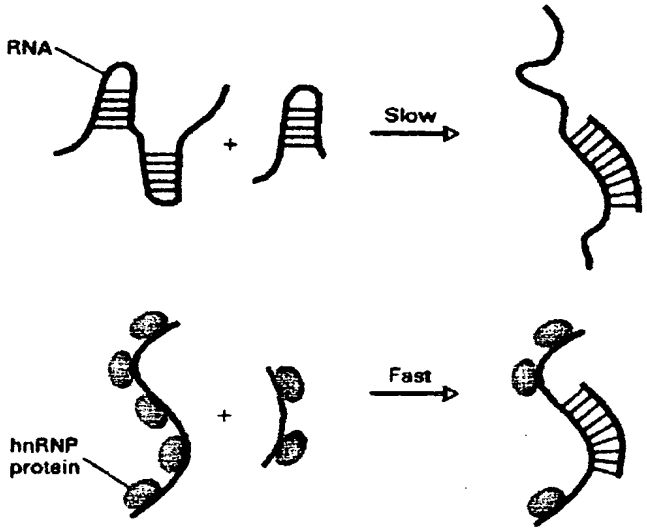

Figure 11-11. Hybridization of RNA molecules in vitro is accelerated by hnRNP proteins. The presence of complex secondary structures within RNA molecules inhibits hybridization between long complementary sequences in separate molecules. Association of hnRNP proteins with RNA is thought to prevent formation of RNA secondary structures, thereby facilitating base-pairing between different complementary molecules. These proteins may have a similar function in vivo. [Adapted from D. S. Portman and G. Dreyfuss, 1994, *EMBO J.* **13**:213.]

EXHIBIT H

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Minireview

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Poly(A) Tail Metabolism and Function in Eucaryotes*

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Recent work on the homopolymeric poly(A) tail of eucaryotic mRNA has led to a clearer understanding of its synthesis, degradation, and function. This review will emphasize the key insights of these studies and attempt to summarize current working models. Two recurring themes in poly(A) research, the recruitment of enzymes by RNA binding proteins and the recognition of degenerate RNA sequences by combinations of RNA binding proteins, will also be highlighted.

Poly(A) Metabolism

Nuclear Polyadenylation—The 3′-ends of mRNA are generated in the nucleus in two steps. The precursor RNA, extending beyond the mature 3′-end for hundreds of nucleotides, is cleaved endonucleolytically at one particular phosphodiester bond. The 3′-OH group of the upstream fragment then receives the poly(A) tail by polymerization from ATP. The cleavage reaction depends on two RNA sequence elements, the highly conserved sequence AAUAAA 10–30 nucleotides upstream of the cleavage site and a poorly defined GU- or U-rich sequence approximately the same distance downstream. In some cases, sequences upstream of AAUAAA increase the efficiency of 3′-end processing. *In vitro*, polyadenylation can be carried out independently of cleavage with precleaved RNA substrates ending at the cleavage/polyadenylation site. This reaction also depends on the sequence AAUAAA (1).

AAUAAA is the binding site for the cleavage and polyadenylation specificity factor (CPSF),[1] which is, like AAUAAA, essential for both steps of the processing reaction. CPSF contains subunits of 160, 100, 73, and, possibly, 30 kDa (2). The largest subunit may have RNA binding activity (3). The sequence element downstream of the cleavage/polyadenylation site appears to be bound by a protein known as cleavage stimulation factor (CStF) or cleavage factor I (CFI) with subunits of 77, 64, and 50 kDa (4, 5). The 64-kDa subunit can be UV-cross-linked to RNA, and its cDNA sequence shows a ribonucleoprotein-type RNA-binding domain (6). CStF/CFI is not involved in polyadenylation.

One or two additional factors, required to reconstitute specific 3′-end cleavage, have not been purified so far. Like CStF/CFI, they are dispensable for polyadenylation. Finally, poly(A) polymerase, the enzyme that synthesizes the poly(A) tail, is also involved in the cleavage reaction. Initially purified as a fully active degradation product of 60 kDa (7), it has been shown by cDNA cloning to exist in at least two forms of 78 and 83 kDa (8, 9).

Prior to the cleavage reaction, processing factors and substrate RNA form a large complex that is detectable after incubation of the RNA in crude nuclear extract with ATP. As the complete set of processing factors is not yet available in pure form, only partial complexes have been reconstituted with purified material. CPSF by itself binds to RNA containing the AAUAAA sequence (3). The CPSF·RNA complex is quite unstable; it is stabilized by CStF/CFI (4, 10). Since this stabilization requires the downstream sequence element, one may infer that CStF/CFI binds to this sequence. Binding of CStF is also enhanced in the ternary complex with CPSF as judged by a more efficient cross-linking of its 64-kDa subunit (11). Binding of CPSF to RNA is also strengthened by poly(A) polymerase, possibly explaining why this enzyme stimulates the cleavage reaction (12). From these data one may infer that the 3′-processing complex contains, in addition to the RNA, at least CPSF, CStF/CFI, and poly(A) polymerase. Unresolved questions include the roles of the still uncharacterized cleavage factors, the identity of the endonuclease, and the role of ATP in complex formation and cleavage.

Once cleavage has occurred, polyadenylation ensues in a closely coupled fashion; the cleaved intermediate is not detectable under normal reaction conditions. The tight coupling is probably due to the presence of poly(A) polymerase in the cleavage complex. With a precleaved substrate, CPSF and poly(A) polymerase are sufficient for poly(A) synthesis. In the absence of CPSF, poly(A) polymerase is not only almost completely inactive but also indifferent to the presence or absence of the AAUAAA sequence in the substrate RNA. The inactivity reflects a poor affinity for RNA; complexes between poly(A) polymerase and RNA can be detected only at high protein concentrations. A ternary complex containing poly(A) polymerase, CPSF, and the substrate RNA can be inferred from its increased stability compared with a CPSF·RNA complex (see above) and its decreased mobility in gel shift experiments. Thus, CPSF recruits poly(A) polymerase to the RNA and makes it specific for RNAs that contain the CPSF binding site, AAUAAA (12).

Polyadenylation by purified poly(A) polymerase and CPSF is strongly stimulated by poly(A) binding protein II (PABII) (13, 14). The nuclear PABII is different from the cytoplasmic poly(A) binding protein I (see below). Once the growing poly(A) tail has reached a length of 10–12 nucleotides, PABII binds and the further rate of extension is strongly increased. Poly(A) synthesis is therefore biphasic, with a slow oligoadenylation phase followed by rapid extension. In the presence of PABII, RNAs that carry an oligo(A) tail capable of binding PABII can be extended independently of CPSF and its binding site AAUAAA. However, extension is much more rapid when both stimulatory factors are present. PABII also stimulates 30–50-fold the extension of a simple poly(A) primer in the absence of CPSF. Like CPSF, PABII appears to act by recruiting poly(A) polymerase to the RNA. A complex containing PABII and poly(A) polymerase on the RNA has not yet been directly demonstrated. However, PABII further stabilizes the complex containing PAP and CPSF, suggesting the existence of a quaternary complex (12).

Changes in the functional properties of poly(A) polymerase are consistent with the hypothesis of quaternary complex formation. On its own, the enzyme is entirely distributive, dissociating from its primer after every polymerization event. In the

* This minireview will be reprinted in the Minireview Compendium, which will be available in December, 1993.

[1] The abbreviations used are: CPSF, cleavage and polyadenylation specificity factor; CStF, cleavage stimulation factor; CFI, cleavage factor I; PABII, poly(A) binding protein II; PAN, poly(A) ribonuclease; UTR, untranslated region; CPE, cytoplasmic polyadenylation element.

presence of either PABII or CPSF, the processivity increases slightly to less than 10 nucleotides polymerized per binding event. Only in the presence of both stimulatory factors can poly(A) polymerase synthesize a full-length poly(A) tail of 200 nucleotides in a single processive event (12).

In the nuclei of mammalian cells, poly(A) tails grow to a length of 200–250 nucleotides before the RNA is exported to the cytoplasm. The *in vitro* system reconstituted from purified CPSF, poly(A) polymerase, and PABII reproduces this length control; processive synthesis stops after the addition of approximately 200 adenylate residues, and only slow, presumably nonprocessive synthesis continues (13). The mechanism inducing this loss of processivity is not understood.

In summary, the apparently simple reaction of cleavage and polyadenylation is carried out by a surprisingly complex set of at least five factors, several of which are composed of multiple subunits. Most of these factors are incorporated into a large complex with the substrate RNA prior to the first catalytic step. Several aspects of this complex may be relevant to other RNA processing reactions. For instance, many factors serve in substrate recognition rather than catalysis. Substrate recognition is achieved through a network of weak interactions rather than by a few high affinity interactions. Such a requirement for the colocalization of many independent factors provides a possible explanation for why mRNA processing signals can be both large in size and degenerate. Similarly, the interactive fashion of substrate recognition also suggests that the requirement for a certain part of a processing signal or a certain factor may depend on the "strength" of the remaining parts of the signal or the concentration of all other factors. Finally, the conventional idea of a linear reaction pathway may not always be appropriate to describe the assembly of a processing complex. Although many binding reactions influence each other, there is no strict dependence of one on the other. Thus, several alternative pathways may lead to the same product, and a preferred pathway established for one substrate under one set of conditions may not hold for a different substrate or a different set of conditions.

Cytoplasmic Deadenylation—Poly(A) tail shortening in the cytoplasm is initiated after nuclear transport, although it is not known if this reaction first requires the association of the mRNA with the translational apparatus (15). Insight into the mechanism of deadenylation was achieved with the observation that deletion of the cytoplasmic poly(A) binding protein (PAB1) from yeast prevented the normal shortening of the cellular poly(A). This result was in direct contrast to the anticipated one of induced tail destabilization since it had been previously thought that mRNA binding proteins protect the RNA from nucleases. Instead, these data suggested that a deadenylase in yeast required PAB1 for function (16).

The yeast poly(A) ribonuclease (PAN) was purified from soluble extracts based upon its ability to degrade poly(A) only in the presence of PAB1 (17). The purified enzyme displays an absolute requirement for a ribonucleoprotein substrate. PAN is normally a distributive exoribonuclease that releases 5′-monophosphates in a Mg^{2+}-dependent reaction. Poly(A) is degraded in two kinetically and biochemically distinct phases. In the shortening phase, PAN degrades poly(A) down to a minimal length of 12–25 adenine residues, a length sufficient to bind one PAB1 molecule (18). Interestingly, PAN can shorten some substrates in the absence of PAB1 as long as an appropriate concentration of spermidine is present. In the terminal deadenylation phase, PAN brings the mRNA to a completely deadenylated form. This second reaction can also occur in the absence of PAB1 at a much slower rate. The temporal separation of the shortening from the terminal deadenylation reaction probably results from the inability of PAB1 to slide or dissociate from the residual poly(A) tail. A recently described mammalian poly(A) nuclease also exhibits a Mg^{2+}-dependent exonucleolytic activity that releases 5′-monophosphates (19). Whether this enzyme requires a ribonucleoprotein substrate for activity is not yet known.

Many similarities exist between the mechanisms of polyadenylation and deadenylation. First, each of the reactions occur in two phases. Second, each of the reactions have a dependence on a poly(A) binding protein that explains their biphasic nature. Finally, each of the PABs is required for the reactions to occur efficiently, suggesting that a key function for the PABs in each of these reactions is the localization of either poly(A) polymerase or PAN to their substrate.

Poly(A) Metabolism in the Oocyte—Animal oocytes contain large amounts of mRNA stored for use during maturation and early embryonic development. Gene expression during these stages can be regulated at the level of translation, and this occurs in many cases through alterations in the poly(A) tail lengths. Tail length is regulated by the control of both its synthesis and degradation (for a more detailed review of the following topics see Ref. 20).

Messenger RNA synthesized in the oocyte before maturation is polyadenylated normally in the nucleus. In the cytoplasm, it is subject to apparently two different deadenylation reactions. Maternal transcripts being translated retain poly(A) tails longer than 50 nucleotides. In contrast, transcripts that are not being translated in the oocyte but which will be subject to regulated polyadenylation and translation following oocyte maturation are deadenylated to an even greater extent. The specific deadenylation of these transcripts appears to result from the presence of a unique sequence in their 3′-UTRs (21).

Following maturation, the translational pattern of the oocyte changes. Some previously active mRNAs are no longer translated as a result of their deadenylation by a potentially different enzyme than that found in the immature oocyte. This poly(A) ribonuclease is activated only after the oocyte's cytoplasmic and nuclear compartments merge due to the maturation-induced germinal vesicle breakdown. This ribonuclease activity does not require specific signals. For instance it will destroy polyadenylated bacterial sequences or even polyadenylated poly(C).

Maturation also induces the translation of previously dormant RNAs, and these are subject to an extension of their poly(A) tail. This developmentally regulated polyadenylation occurs in the cytoplasm, in contrast to the nuclear 3′-processing reactions. The reaction depends upon a sequence related to UUUUUUAU, the cytoplasmic polyadenylation element (CPE), and it also requires the AAUAAA polyadenylation signal. RNAs that have both a CPE and AAUAAA receive a poly(A) tail upon oocyte maturation; all others lose their tail. Polyadenylation thus seems to be a positively regulated event overriding the default deadenylation reaction. The implication of this is that changes in poly(A) tail lengths in the developing organism result from regulated adenylation and not deadenylation. However, the possibility still exists that the deadenylation of some mRNAs is induced by a unique deadenylation element.

CPE-dependent polyadenylation can be carried out in *Xenopus* egg extracts. Their fractionation demonstrated a poly(A) polymerase activity as well as an RNA binding activity specific for both AAUAAA and the CPE. It is the RNA binding activity that is induced by oocyte maturation (22). In contrast, poly(A) polymerase activity is similar before and after maturation. Surprisingly, CPE-dependent polyadenylation can be reproduced *in vitro* with recombinant mammalian poly(A) polymerase and purified mammalian CPSF. Direct experiments suggest that CPSF has a binding specificity not only for AAUAAA but also

for a CPE-like sequence.[2] Thus, it is possible that cytoplasmic polyadenylation is catalyzed by essentially the same components as the nuclear reaction. Additional factors may modify the basic reaction. It is also possible that the sequences upstream of AAUAAA, which influence the polyadenylation of a number of genes in mammalian cells, function like the CPE in enhancing the binding of CPSF.

Poly(A) Function

Understanding the function of the poly(A) tail remains a major goal. Significant progress in two areas, mRNA degradation and translation initiation, has shed light on the role of the polymer and highlighted a fundamental theme in RNA metabolism, that of ribonucleoprotein recognition by trans-acting factors. Areas that remain less well defined but which could also require the poly(A) tail are intracellular mRNA localization and mRNA nuclear transport.

mRNA Degradation—The destruction of eucaryotic mRNA is a highly regulated process that occurs at rates differing by over 2 orders of magnitude (for a detailed review, see Ref. 23). The information that determines at what rate an mRNA is to be destroyed lies within destabilizing determinants throughout the message. While examples of destabilizing sequences abound, the only sequences known to stabilize mRNA directly are the cap structure and the poly(A) tail. It is this property of stabilization that makes poly(A) tail removal a target for the activity of the destabilizing sequences elsewhere on the mRNA.

That poly(A) tail removal occurs before mRNA is degraded has been shown for several different messages with essentially the same technique (23). In these experiments, mRNA synthesis is initiated and then abruptly inhibited. The bolus of RNA that is produced at first carries the long nuclear poly(A) tail, and once transported to the cytoplasm it begins to lose its tail. The degradation of the mRNA is found to begin only after the poly(A) tail has been removed. For example, mRNAs that are rapidly degraded first lose their tails rapidly, while those that are slowly degraded first lose their tails slowly. Furthermore, mutations that alter the rate of mRNA deadenylation also change the rate of degradation.

Each of these experiments has contributed to the working model that destabilizing sequences function by stimulating deadenylation. This stimulation can be for either the shortening or terminal deadenylation phase, or both. A closer examination of the available data suggests that the mRNA need not be completely deadenylated before the next phase of the degradation reaction occurs (24). Instead, it seems that shortening of the oligoadenylate tail to a length below approximately 10 nucleotides, a length incapable of high affinity binding to PAB1, is sufficient to induce the next step. Whether the next step in the decay reaction is also common to all mRNAs or whether it is at this juncture that each mRNA is destroyed by separate pathways remains to be determined.

3′-UTR-dependent deadenylation utilizing the purified yeast PAN has been reconstituted, providing some mechanistic data about the action of these elements (18). For instance, it is clear that *in vitro* the terminal deadenylation step occurs at different rates for different mRNAs and that the determinants for these rates can lie within the 3′-UTR of the mRNAs. Furthermore, the identification of an mRNA sequence that completely inhibits terminal deadenylation *in vitro* raises the possibility that mRNA can be stabilized *in vivo* by similar sequences.

Finally, *in vitro* deadenylation studies on the yeast MFA2 3′-UTR reveal that it stimulates deadenylation of the mRNA by inducing PAN processivity (18). This simple switch of enzyme mechanism has important implications for studying the mode of action of the destabilizing elements. It provides support for the simple model that all destabilizing sequences operate by targeting association factors for PAN to the mRNA. As a result, biochemical dissection of the mechanism of action of these elements could utilize changes in PAN activity as an enzymatic assay. In general, the regulation of mRNA decay by changes in PAN processivity is a recapitulation of the recurring theme of differential affinity of enzymes for their substrate induced by ribonucleoprotein complexes.

Translation Initiation—Subsequent to the discovery of the poly(A) tail, the potential role for this sequence in mRNA translation was explored and then dismissed based upon the data that poly(A)-deficient mRNA was translated *in vitro* almost as well as polyadenylated mRNA (reviewed in Ref. 25). However, based on the recent observations summarized below, it is now generally accepted that mRNA translation is most efficient with, and sometimes even completely dependent upon, the poly(A) tail (for a more detailed treatment, see Ref. 26).

Investigations into the cytoplasmic polyadenylation and deadenylation reactions in the maturing oocyte support the hypothesis that the purpose of controlling the adenylation status of the mRNA is to control its translatability (reviewed in Ref. 20). Mutations that destroyed the CPE on a maternal mRNA prevented both its polyadenylation and translation. Furthermore, if an mRNA that was normally deadenylated and translationally repressed during maturation had inserted within its 3′-UTR a CPE, it remained polyadenylated as well as translationally activated. Proof that the translational recruitment was specific for the poly(A) tail and not the presence of a CPE came with the observation that a CPE containing mRNA modified with a dideoxyadenosine at its 3′-end was neither translationally competent nor capable of cytoplasmic polyadenylation. Finally, for several mRNAs, *in vitro* polyadenylation followed by oocyte injection was sufficient to induce translation at an inappropriate time.

Independent genetic data from the yeast *Saccharomyces cerevisiae* also support the conclusion that poly(A) is necessary for translation *in vivo* (reviewed in Ref. 15). In these experiments, temperature-sensitive mutations in the essential yeast *PAB1* gene were found to cause an inhibition of translation at the restrictive temperature. The accumulation of ribosomal subunits at this temperature indicated that the inhibition of translation was due to a block in initiation. Support for this hypothesis came from an examination of suppressors of a *PAB1* deletion. Each of these suppressor mutations decreased the level of the 60 S ribosomal subunits. One of the mutations resulted in the loss of the large ribosomal subunit protein L46, while another altered a polypeptide with significant homology to a family of RNA-dependent ATPases that appeared to be involved in ribosomal RNA maturation. These data confirmed the conclusion that the primary deficiency in *pab1* mutants was translational and that a 60 S subunit-dependent step in the translation initiation pathway was affected.

Further support for this conclusion came from an analysis of the mild stimulation of translation *in vitro* by poly(A) (27). This study found that the 60 S subunit joining step was more efficient when the mRNA was polyadenylated. More recently, genetic interactions between the yeast SIS1 protein required for translation (28) and PAB1 have enlarged the list of potential players mediating the translational requirement for the poly(A) tail. Finally, the discovery that PAN, the PAB1-activated ribonuclease, is also required for translation initiation (17) creates a new series of questions regarding the relationship between the mRNA degradation and translation initiation reactions.

[2] A. Bilger, C. Fox, E. Wahle, W. Keller, and M. Wickens, manuscript in preparation.

Summary and Perspective

The detailed examination of poly(A) tail synthesis, degradation, and function has yielded important insights. Enzyme recognition of ribonucleoprotein substrates and alterations in enzyme mechanism due to variations in substrate affinity are two important examples. The utilization of the poly(A) tail in multiple reactions shows that a single RNA sequence can be recognized by different groups of enzymes. The requirement for poly(A) in each of these reactions explains why sequence elements within mRNA that control poly(A) tail metabolism have such a dramatic effect on mRNA expression.

Future work in poly(A) research will almost certainly include a more detailed analysis of both the adenylation and deadenylation reactions, the identification of more sequence elements that can regulate them, and the reconstitution of many of the poly(A)-dependent events within the cell. With this should come an even greater appreciation for the mechanisms used in the post-transcriptional control of gene expression.

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Role of Polyadenylation in Nucleocytoplasmic Transport of mRNA

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To examine the role of polyadenylation in the nuclear export of mRNA, we have replaced the poly(A) signal in a Rev-responsive human immunodeficiency virus type 1-based reporter gene with a *cis*-acting hammerhead ribozyme. Transcripts from this gene thus acquire a 3′ terminus by *cis*-ribozyme cleavage rather than by polyadenylation. The nuclear and cytoplasmic distribution of transcripts was investigated using transient gene expression and quantitative RNase protection assays. In the absence of Rev, a basal level of polyadenylated unspliced mRNA transcribed from a poly(A) signal-containing control reporter gene was detected in the cytoplasm of transfected COS7 cells. However, cytoplasmic ribozyme-cleaved unspliced RNA was only barely detectable. The nuclear/cytoplasmic (n/c) ratio of polyadenylated RNAs was 3.8, while the n/c ratio for ribozyme *cis*-cleaved RNAs was 33. The cytoplasmic localization of the polyadenylated unspliced mRNA was enhanced about 10-fold in the presence of Rev and the Rev-responsive element. In marked contrast to this, ribozyme-cleaved RNA accumulated almost exclusively (n/c ratio of 28) in the nucleus in the presence of Rev. Actinomycin D time course analysis suggested that the low levels of the cytoplasmic ribozyme-cleaved RNAs in both the presence and absence of Rev were due to severe export deficiency of ribozyme-cleaved RNA. Finally, by inserting a 90-nucleotide poly(A) stretch directly upstream of the ribozyme cassette, we have demonstrated that a long stretch of poly(A) near the 3′ end of a ribozyme-cleaved transcript is not sufficient for directing mRNA export. Taken together, these results suggest that polyadenylation is required for the nucleocytoplasmic transport of mRNA and that Rev interaction with the Rev-responsive element cannot bypass this requirement.

Messenger RNA precursors (pre-mRNAs) are synthesized in the nucleus by RNA polymerase II and then subjected to a series of processing reactions which include the addition of a 7-methylguanosine cap at the 5′ end, the removal of introns by splicing, and the generation of mature 3′ ends. With the exception of histone genes, whose transcripts end in a highly conserved stem-loop structure (for reviews see references 2 and 36), the 3′ end is formed by cleavage and polyadenylation (recently reviewed; see reference 47). Mature RNA molecules are then translocated to the cytoplasm through the nuclear pore complex (see reference 12 for review). The mechanism of mRNA nuclear export is poorly understood, although this process is fundamental to eukaryotic gene expression. Recently, however, evidence has begun to accumulate that capping and subsequent processing of pre-mRNA to generate mature mRNA influence the efficiency of nuclear export (reviewed in references 12, 20, and 26).

Evidence that the 5′ monomethylguanosine cap structure may serve as a positive signal for mRNA nuclear export was derived from microinjection experiments carried out with *Xenopus* oocytes (9, 18, 21). It was shown that mRNAs carrying the normal 5′ cap structure were rapidly exported to the cytoplasm after being microinjected into oocyte nuclei, while mRNAs containing a different cap structure were delayed for export. On the other hand, studies of mRNA export in yeast and mammalian cells have implicated splicing components in the nuclear retention of pre-mRNA (5, 27). Mutations that allowed spliceosomes to form but that prevented splicing led to the retention of pre-mRNAs in the nucleus. Conversely, mutations that blocked the assembly of spliceosomes induced the cytoplasmic accumulation of unspliced mRNA. Therefore, removal of the splicing machinery from RNAs may be a prerequisite for export.

In contrast to the negative effects of spliceosomes on mRNA export, a positive link between 3′-end processing and the export of intronless mRNAs has been established (10). When an intronless, prokaryotic neomycin resistance gene was introduced into mammalian cells, cleavage/polyadenylation or histone 3′-end processing stimulated the egress of mRNA from the cell nucleus. However, when 3′ ends were formed by *cis*-acting ribozyme cleavage in vivo, nucleocytoplasmic transport did not occur. It was not clear from these studies, however, whether polyadenylation is of general importance for the export of mRNA, or whether a poly(A) sequence at or near the 3′ end is sufficient for promoting mRNA export.

Virus-encoded proteins involved in the regulation of viral gene expression have proven to be useful probes for cellular functions involved in nucleocytoplasmic mRNA export. Study of the function of the human immunodeficiency virus type 1 (HIV-1) Rev protein in the regulation of gene expression has provided especially useful insights. Rev facilitates the cytoplasmic transport of unspliced and partially spliced viral RNAs by binding to the Rev-responsive element (RRE) present in its target RNA molecules (5, 11, 13, 14, 34; for a review, see reference 7). Two major models have been proposed to explain how Rev controls mRNA export. The first suggests that Rev stimulates RNA export by displacing cellular splicing components from viral pre-mRNA (5, 24, 30). The second model suggests that Rev directly promotes export of pre-mRNA through a pathway distinct from that used for normal cellular mRNA (15, 16, 23, 33, 37, 45, 46). Of particular interest are results of a recent microinjection study performed with *Xenopus* oocytes (16). In that work it was reported that Rev can induce export of RRE-containing RNAs (including intron lari-

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ats) in the absence of polyadenylation. These data raised the possibility that the Rev function may bypass the possible requirement of polyadenylation for RNA export in *Xenopus* oocytes. Whether this is also the case for mRNA transport in mammalian cells has not yet been established.

We have been interested in the role of polyadenylation in mRNA nuclear export. Using a *cis*-acting ribozyme that uncouples 3′-end formation from the mammalian cellular polyadenylation machinery, we offer evidence here that polyadenylation is a prerequisite for the export of some HIV-related transcripts in mammalian cells. This hypothesis is further supported by the observations that Rev/RRE cannot stimulate the nuclear export of ribozyme *cis*-cleaved RNA as efficiently as it stimulates the export of polyadenylated mRNA and that a long stretch of poly(A) near the 3′ end is not sufficient for directing mRNA export, whether in the presence or absence of Rev/RRE. Finally, other results reported here reveal that Rev stabilizes ribozyme-cleaved, RRE-containing RNA in the nuclear compartment.

MATERIALS AND METHODS

Restriction enzymes, T4 DNA ligase, and DNA polymerase I large fragment were from New England Biolabs and were used as suggested by the manufacturer. RNase T_1/T_2 was prepared as previously described (28). [α-^{32}P]UTP was from New England Nuclear. RQ1 DNase I and T3 and T7 RNA polymerases were from Promega. All recombinant plasmids were propagated in *Escherichia coli* JM83. Actinomycin D was from Sigma.

Cell culture and transfection. COS7 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Transfections were carried out with a modified $CaPO_4$ DNA coprecipitation method (4). Briefly, 4 μg of the indicated reporter plasmid along with 10 μg of the PyE (see below) expression vector was transfected into COS7 cells, with or without the cotransfection of Rev expression plasmid pRSV-Rev (obtained from Y. Luo and B. M. Peterlin). The molar ratio of the reporter plasmid and pRSV-Rev was 1:4. This ratio has previously been shown to provide optimal Rev effects (19). The total amount of DNA used for transfection per 15-cm plate was adjusted to 30 μg with pUC18.

Constructs. Plasmid pDM128 (19, 41, and Fig. 1) was obtained from Y. Luo and B. M. Peterlin. It derives from the HIV-1 Env region, contains a single HIV-1 intron which is flanked by the HIV-1 5′ and 3′ splice sites, and harbors the RRE. Transcription is driven by the simian virus 40 (SV40) early promoter/enhancer, and 3′ ends are formed by the HIV-1 poly(A) signal. For plasmid pDM128RZ, the *Eco*RI site within the intron of pDM128 was destroyed by partial *Eco*RI digestion followed by filling in ends using Klenow enzyme and religation. This rendered the *Eco*RI site proximal to the polyadenylation signal unique. This plasmid was digested with *Afl*II and *Eco*RI (removing the entire HIV-1 polyadenylation signal), and a 282-nucleotide (nt) *Dpn*I fragment comprising a ribozyme sequence from BS-RZ-2 (29) was inserted. Finally, a deletion from *Eco*RV to *Eco*RI (Fig. 1) was introduced to shorten the distance between the ribozyme cleavage site and the 3′ splice site to give pDM128RZ. To create the riboprobe used for all experiments, pDM128RZ was digested with *Bgl*II and the ends were made blunt and then digested with *Apa*I. The resulting *Apa*I to *Bgl*II fragment (1,758 nt) was then inserted into pBlueScript opened with *Apa*I and *Sma*I. The 818-nt riboprobe (Fig. 1) was synthesized with this plasmid as a DNA template for in vitro runoff transcription. Construct pDM128RZA was built by inserting a 90 bp-poly(dA/dT) fragment with *Eco*RI sites at both ends (43) directly upstream of the ribozyme cassette at the *Eco*RI site of pDM128RZ. Transcripts expressed from this vector possess a 90-nt poly(A) sequence near their 3′ ends, followed by a 31-nt non-A extension. Plasmid pRSV-Rev contains the Rev coding region from HIV-1 and is driven by the Rous sarcoma virus long terminal repeat promoter (6). PyE is the entire polyomavirus genome cloned into pUC18 at the *Bam*HI site. It expresses functional polyomavirus early mRNAs.

RNA preparation. RNAs were isolated 48 h after transfection by techniques as described previously (29). To prevent ribozyme cleavage in vitro, extreme care was taken during the preparation of RNA. Mg^{2+} ions, which are essential for ribozyme activity (8), were never present during either the isolation or the analysis procedures. In addition, cell fractionations were carried out on ice and the exposure of cells or cell nuclei to lysis buffer was minimized (less than 2 min on ice). Finally, in the preparation of nuclear, cytoplasmic, and total RNA we used solutions containing 4 M guanidinium thiocyanate, a strong secondary structure denaturant. For the preparation of cytoplasmic RNA, cells were rinsed free of media with ice-cold phosphate-buffered saline and were then disrupted with a modified Nonidet P-40 (NP40) lysis buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.6, 10 mM NaCl, 3 mM $CaCl_2$, 0.5% NP40) on ice for 30 s. Cytoplasmic lysates were collected in new tubes, and appropriate amounts of guanidinium thiocyanate crystals were added to give a final concentration of 4 M. Cytoplasmic RNA was then purified through CsCl step gradients. For nuclear RNA, the above intact cell nuclei which were still attached to the plates were rinsed with ice-cold NP40 buffer twice, followed by lysis in 4 M guanidinium isothiocyanate–20 mM sodium acetate, pH 5.2–0.1 mM dithiothreitol–0.5% *N*-lauryl sarcosine. The RNA was then pelleted through cesium chloride as described above. To isolate total RNA, cells were rinsed free of media and lysed directly with the same guanidinium solution as for nuclear RNA. RNA was then pelleted through CsCl. To perform the actinomycin D time course analysis, cells were exposed to a 5-μg/ml final concentration of actinomycin D for 0, 60, or 120 min before RNA harvest. Forty-eight hours after transfection, RNAs were isolated by procedures described above.

RNase protection assays. Internally labeled RNA probes were made by in vitro transcription by T3 or T7 RNA polymerase in the presence of [α-^{32}P]UTP. DNA templates were removed by RQ1 DNase digestion followed by phenol-chloroform extraction. Internally labeled riboprobes were hybridized in a buffer lacking magnesium ions at 57°C overnight, as described previously (12). The hybridization products were digested with a T_1/T_2 mixture in a buffer lacking Mg^{2+} (28) at 37°C for 2 h, and the resulting samples were resolved on 6% denaturing polyacrylamide gels. Routinely, 10 μg of nuclear, cytoplasmic or total RNA samples were used for RNase protection, except that in the actinomycin D time course analysis of cytoplasmic RNAs, 30 μg of the RNA samples were used for protection. For cytoplasmic pDM128RZ RNAs, films were exposed three times longer.

Quantitation of RNase protection data. Protected bands were quantitated by using a Packard Instant Imager. Background was subtracted by using regions of identical sizes located immediately below each of the experimental bands. The nuclear/cytoplasmic (n/c) ratio of the internal control PyE spliced RNAs served as a useful way to accurately normalize experimental band results. In different experiments, the n/c ratio of spliced PyE mRNA varied between 0.50 and 0.55. We arbitrarily chose 0.50 as a standard ratio for normalization purposes. Thus, the experimental radioactive counts of spliced PyE mRNAs in Fig. 3B, lane 2, were reduced by 10%, to achieve a ratio of 0.50. This same 10% reduction was then applied to the count values for the corresponding experimental bands, Fig. 3A, lane 2. Similar corrections were performed for other RNase protection data.

RESULTS

Experimental strategy. To investigate the effect of polyadenylation on mRNA nucleocytoplasmic transport, we compared the export of a polyadenylated control reporter gene transcript with that of gene transcripts whose 3′ ends were generated by *cis*-acting ribozyme cleavage, in both the presence and absence of HIV-1 Rev. Constructs used are shown in Fig. 1. Plasmid pDM128 served as a positive control for nuclear and cytoplasmic localization and has been described previously (19). It derives from the Env region of HIV-1, contains a single HIV-1 intron which is flanked by HIV-1 5′ and 3′ splice sites and harbors the RRE. Transcription is driven by the SV40 early promoter/enhancer, and 3′ ends of the transcripts are processed by the HIV-1 polyadenylation signal. In pDM128RZ the HIV-1 poly(A) signal of pDM128 has been replaced by a *cis*-acting hammerhead ribozyme and a deletion has been made to shorten the distance between the ribozyme cleavage site and the 3′ splice site. This allowed us to detect both splicing and ribozyme cleavage with a single riboprobe. A consensus histone 3′ stem-loop sequence lacking the CAGA processing signal was inserted upstream of the ribozyme to stabilize the ribozyme-cleaved RNA should it be transported to the cytoplasm (10). Thus, construct pDM128RZ was expected to generate RNA transcripts which end in a histone 3′ stem-loop structure but which are not produced by the histone 3′-end processing mechanism. Finally, in an attempt to determine whether the poly(A) sequence at the 3′ end of mRNAs is sufficient for export, a stretch of 90 consecutive A residues was inserted directly upstream of the histone stem-loop structure and the hammerhead ribozyme to give pDM128RZA. Transcripts generated by this gene have poly(A) stretches near their 3′ ends, but this poly(A) is not produced by the polyadenylation machinery.

To assess nuclear export of mRNAs, the above indicated plasmids were each transfected into COS7 cells along with a plasmid expressing the PyE, with or without cotransfection of pRSV-Rev (6), which expresses the wild-type HIV-1 Rev pro-

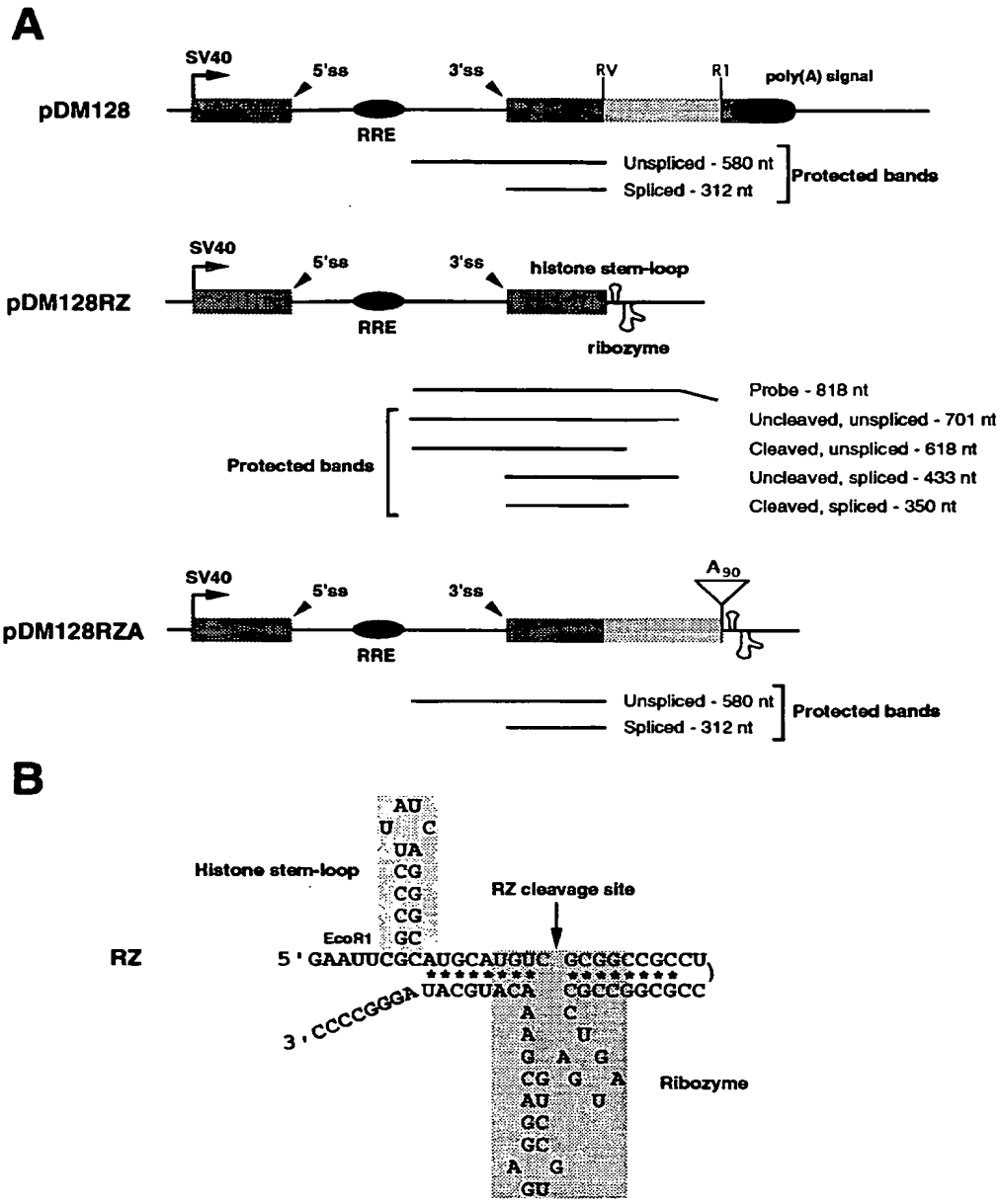

FIG. 1. Constructs used in this study. (A) Transcription from the three constructs is driven by the SV40 early promoter/enhancer, which is labeled by an arrowhead. Thin lines denote intron sequences, with the RRE indicated. Darkly stippled boxes represent exons, and the lightly stippled box depicts sequences missing in construct pDM128RZ. The HIV-1 poly(A) signal is marked by the solid ellipse. The 5' and 3' splice sites (5'ss and 3'ss) and two important restriction sites are also shown. RV, *Eco*RV; RI, *Eco*RI. In plasmid pDM128RZ, the *cis*-acting hammerhead ribozyme and the upstream histone 3' stem-loop structure are indicated. In construct pDM128RZA, the 90-nt poly(A) insertion is marked by a triangle. The 818-nt riboprobe generated by HIV-RZ (see Materials and Methods) and the predicted protection fragments are shown. Constructs are not drawn to scale. (B) Schematic representation of the ribozyme cassette. The histone stem-loop and the hammerhead ribozyme portions are highlighted. The ribozyme cleavage site is 9-nt downstream of the histone stem-loop.

tein. Expression of polyomavirus early mRNAs served as an internal control for transfection efficiency, nuclear and cytoplasmic fractionation of RNA (unspliced polyomavirus early transcripts are exclusively confined to the nucleus; data not shown, and see below), and RNA loading and as a standard for RNA stability assays. Use of this internal control thus made it possible to draw both qualitative and quantitative conclusions with respect to expression of transcripts in transfected cells. Forty-eight hours after transfection, nuclear and cytoplasmic RNAs were isolated and the intracellular distribution of RNAs was analyzed by quantitative RNase protection assays.

A hammerhead ribozyme is active in vivo. In previous stud-

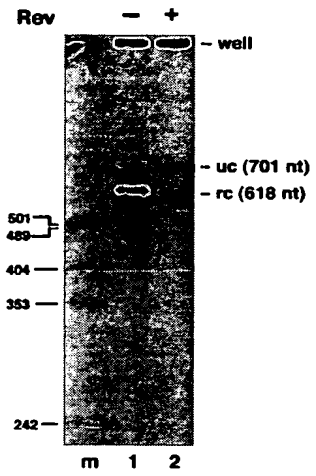

FIG. 2. A hammerhead ribozyme is active in vivo. Plasmid pDM128RZ was transfected into COS7 cells with (lane 2) or without (lane 1) cotransfection of pRSV-Rev. Total RNAs were prepared and were analyzed by RNase protection. uc, ribozyme-uncleaved unspliced RNA; rc, ribozyme-cleaved unspliced RNA; m: molecular size marker in nucleotides. The 818-nt riboprobe shown in Fig. 1 was used for RNase protection assays. The lower levels of RNA in lane 2 appear only because less RNA was loaded onto gel.

ies of polyomavirus gene expression, we exploited a *cis*-acting hammerhead ribozyme to uncouple 3′-end formation from the cellular polyadenylation machinery. It was found that viral RNAs whose 3′ ends were generated by ribozyme cleavage failed to accumulate in the cytoplasm (29 and unpublished data). Here we used the same ribozyme construct to investigate effects of polyadenylation on export of HIV-based gene transcripts.

Unambiguous interpretation of results obtained with constructs encoding a self-cleaving ribozyme are only possible if cleavage occurs in vivo and not during in vitro manipulation. Therefore, before approaching the issue of ribozyme-cleaved RNA nuclear export, we performed a series of control experiments. Figure 2 shows some of these results. To measure ribozyme cleavage in vivo and to examine possible effects of Rev on ribozyme activity, pDM128RZ was transiently transfected into COS7 cells with or without cotransfection of pRSV-Rev. Total RNAs were isolated and assayed by RNase protection using the 818-nt riboprobe capable of distinguishing between ribozyme-uncleaved unspliced, ribozyme-cleaved unspliced, ribozyme-uncleaved spliced, and ribozyme-cleaved spliced RNA species (Fig. 1 and 2). All experiments were carried out under conditions that prevent ribozyme cleavage in vitro (see Materials and Methods). For example, to extract total RNA, cells were lysed with 4 M guanidinium lysis buffer (low pH) followed by ultracentrifugation of the guanidinium lysate on CsCl step gradients to separate RNA from other cellular macromolecules. Particular care was taken to ensure that Mg^{2+}, which is crucial for ribozyme activity, was never present during RNA isolation and protection assays. As demonstrated in Fig. 2, the riboprobe protected two fragments. The 701-nt fragment represents ribozyme-uncleaved unspliced transcripts. The 618-nt fragment indicates ribozyme-cleaved unspliced RNA. Other fragments with the predicted mobilities for spliced products were not detected, indicating that splicing occurred extremely poorly, if at all, in the absence of polyadenylation (see Discussion). Quantitation of the two protected fragments (701 and 618 nt) by using a Packard Instant Imager and correcting for uridine content of the two species revealed that the efficiency of ribozyme cleavage in vivo is about 75%. Similar cleavage efficiencies in the presence and absence of Rev were observed (compare Fig. 2, lanes 1 and 2), indicating that Rev does not affect ribozyme activity in vivo.

RNAs whose 3′ ends are formed by ribozyme cleavage and not by polyadenylation are defective for nuclear export. To determine whether polyadenylation is required for nucleocytoplasmic transport of mRNA, pDM128 and pDM128RZ (which lacks a polyadenylation signal) were each transfected into COS7 cells along with plasmid PyE. Nuclear and cytoplasmic RNAs were isolated 48 h after transfection, and the intracellular localization of the transcripts was assessed by quantitative RNase protection assays. PyE RNAs served both as transfection controls to normalize RNA levels and as controls for efficient nuclear and cytoplasmic RNA fractionation. Unspliced PyE RNA is exclusively retained in the nucleus; its appearance in the cytoplasmic fraction is an indicator of leakage during subcellular fractionation. As before, all experiments were performed under conditions avoiding ribozyme cleavage in vitro (10 and Materials and Methods).

As shown in Fig. 3A, a basal level of unspliced mRNA generated from pDM128 accumulated in the cytoplasm of cells. Unspliced RNA (us, lanes 1 and 2) was distributed in a ratio of about 3.8:1 between the nuclear and cytoplasmic compartments (n/c ratio) while the spliced mRNA has an n/c ratio of 0.26:1, indicating more efficient export of spliced RNA. These observations are consistent with published data (5, 11, 13, 14, 34). In contrast, however, ribozyme-cleaved unspliced RNA (rc, lanes 3 and 4) was almost exclusively restricted to the nucleus with an n/c ratio of 33:1. Only after prolonged autoradiographic exposure was a small amount of rc RNA detected in the cytoplasm (data not shown). The 818-nt riboprobe again detected only two predominant RNA species (lanes 3 and 4): ribozyme-uncleaved unspliced (uc) and ribozyme-cleaved unspliced (rc) RNAs, similar to the results shown in Fig. 2 (see Discussion). Unspliced early-strand RNA transcribed from the control plasmid PyE was never found in the cytoplasm (n/c

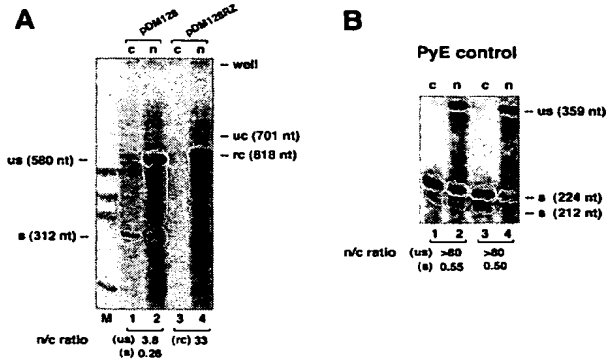

FIG. 3. Export deficiency of ribozyme-processed RNA. Plasmids pDM128 and pDM128RZ together with plasmid PyE were each transfected into COS7 cells. The subcellular distribution of RNAs was examined by quantitative RNase protection. (A) Expression of pDM128 and pDM128RZ by using the 818-nt riboprobe. uc, ribozyme-uncleaved unspliced RNA; rc, ribozyme-cleaved spliced RNA; c, cytoplasm; n, nucleus; m, molecular size marker (same as in Fig. 2). n/c, RNA distribution ratio between the nuclear and cytoplasmic compartments. The n/c ratios have been corrected by the levels of spliced PyE RNAs shown in panel B. (B) Expression of internal control plasmid PyE by using a polyomavirus early region-specific riboprobe.

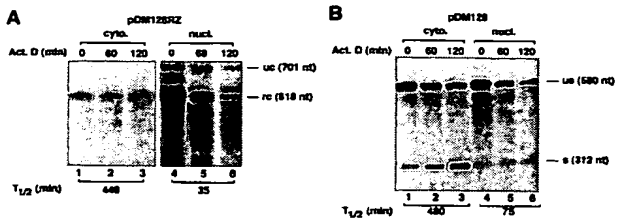

FIG. 4. Actinomycin D (Act. D) time course analysis of nuclear and cytoplasmic pDM128 and pDM128RZ RNAs. Plasmids pDM128RZ (panel A) and pDM128 (panel B) were each transfected into COS7 cells along with PyE. Nuclear and cytoplasmic RNAs were extracted 0, 60, and 120 min after treatment with a 5-μg/ml final concentration of actinomycin D and were analyzed by quantitative RNase protection. For cytoplasmic RNAs, three times more RNA samples were used in RNase protection. For pDM128RZ RNA, radioactive exposure was three times longer. The half-life ($T_{1/2}$) times given have been normalized as described in the text to the levels of the internal control PyE spliced mRNAs (data not shown). uc, ribozyme-uncleaved unspliced RNA; rc, ribozyme-cleaved unspliced RNA; us, unspliced mRNA; s, spliced mRNA; cyto., cytoplasm; nucl., nucleus.

ratio, >80), and there was minimal variation in the pattern of distribution of spliced PyE transcripts between the two cellular compartments (Fig. 3B). This indicates that clean subcellular fractionation was achieved and there was no detectable leakage of nuclear RNA to the cytoplasm.

The dramatic differences in distribution of ribozyme-cleaved unspliced RNA and unspliced RNA from pDM128 between the two cellular compartments suggests that mRNAs that are not polyadenylated are defective in nuclear export. To rule out the possibility that the low level of ribozyme-cleaved unspliced RNAs seen in the cytoplasm might result from rapid degradation, we measured half-life times of the two RNA species in both the nuclear and cytoplasmic compartments. Quantitative RNase protection assays following actinomycin D treatment of the transfected cells were performed. Nuclear and cytoplasmic RNAs were isolated from cells transfected with the indicated reporter plasmids together with plasmid PyE. As shown in Fig. 4, rc RNA is relatively stable, with a half-life time of 440 min, similar to that of us RNA from pDM128 (compare Fig. 4A, lanes 1 to 3, with Fig. 4B, lanes 1 to 3). However, the half-life time of rc RNA in the nucleus is only 35 min (Fig. 4A, lanes 4 to 6). These results showing different half-lives for rc species in the nuclear and cytoplasmic compartments support the idea that the small amount of rc RNA detected in the cytoplasm is due to poor export rather than to leakage. They also suggest little or no nuclear leakage. It is not clear, however, whether the cytoplasmic rc RNAs derived from true export of nuclear rc RNAs or from export of cryptically polyadenylated RNA followed by *cis*-ribozyme cleavage in the cytoplasm.

Interestingly, in Fig. 4B, lanes 1 to 3, right panel, the level of cytoplasmic s RNA increases during the assay period, in contrast to that of the us RNA. This suggests that the export rate of spliced RNA from the nucleus to the cytoplasm exceeds that of degradation in the cytoplasm. This also indicates that actinomycin D does not block nuclear export. A similar pattern is seen for the nuclear spliced RNA, possibly due to cytoplasmic contamination. On the basis of the above data, we conclude that polyadenylation is required for mRNA nuclear export.

Rev cannot enhance export of ribozyme-cleaved RNA as efficiently as it enhances export of polyadenylated mRNAs. It has been well established that Rev enhances nucleocytoplasmic transport of RRE-containing pre-mRNAs (5, 11, 13–16, 23, 33, 34, 37, 46). However, the mechanism of this action is not yet clear. Recently, evidence favoring the mode of direct action of Rev has begun to accumulate. First, in experiments with T lymphocytes (33) and *Xenopus* oocytes (16), it has been shown that an increased cytoplasmic localization of RRE-bearing mRNAs in the presence of Rev was not accompanied by a decreased level of fully spliced mRNAs in the cytoplasm. Second, Rev has been found to be a shuttling protein which may be associated with its target mRNAs during export (23, 37). Third, microinjection experiments (16) have shown that Rev promotes export of RRE-containing RNAs, including that of lariat introns, from the microinjected oocyte nuclei in the absence of polyadenylation. Finally, a number of cellular factors which interact with Rev have recently been identified (3, 15, 17, 45, 46), with some being components of or associated with nuclear pore complexes (3, 15, 17, 46).

To determine whether polyadenylation is required for Rev to stimulate export of RRE-containing mRNA in a mammalian system, we performed the following experiments. pDM128 and pDM128RZ were each transfected into COS7 cells, together with the PyE plasmid and the Rev expression vector. Nuclear and cytoplasmic fractionation and RNase protection assays were carried out as described above. Results are presented in Fig. 5. As expected, Rev significantly increased the cytoplasmic localization of polyadenylated unspliced mRNA (us) (compare Fig. 5A, lanes 1 and 2, to Fig. 3A, lanes 1 and 2). The n/c ratio of us RNA switched from 3.8:1 in the absence of Rev to 0.4:1 in the presence of Rev. However, the n/c ratios of spliced pDM128 RNAs are similar in the both situations. This finding is in agreement with previously published results (5, 11, 13, 14, 34). Surprisingly, the nuclear and cytoplasmic distribution pattern of the ribozyme-cleaved unspliced RNA (rc) remains unchanged (compare Fig. 5A, lanes 3 and 4, to Fig. 3A, lanes 3 and 4). The simplest interpretation of these data is that Rev does not act independently of polyadenylation in this system. Should Rev stimulate export of mRNA independently of polyadenylation, a distribution pattern of rc RNA similar to that of us RNA from pDM128 in the presence of Rev should have been observed. However, this was not the case.

We next performed actinomycin D chase experiments to analyze the stability of rc RNAs in both cellular compartments. As shown in Fig. 6, lanes 1 to 3, cytoplasmic rc RNA has a half-life similar to that of polyadenylated unspliced pDM128 mRNA (Fig. 4A, lanes 1 to 3). This indicates that the low level

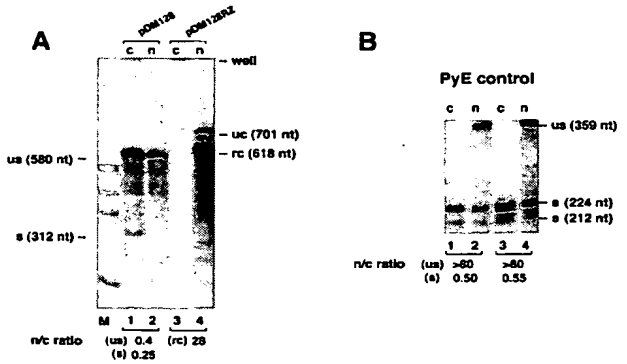

FIG. 5. Rev/RRE cannot bypass the requirement of polyadenylation for nuclear export of mRNAs. Plasmids pDM128 and pDM128RZ, together with plasmid PyE, were each transfected into COS7 cells, along with cotransfection of plasmid pRSV-Rev. Nuclear and cytoplasmic RNAs were isolated and quantitative RNase protection assays were performed. Labels are the same as in Fig. 3. (A) RNase protection assays of pDM128 and pDM128RZ; (B) RNase protection assays of the internal PyE control transcripts.

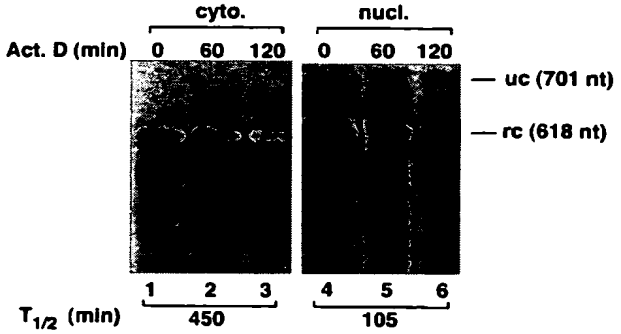

FIG. 6. Actinomycin D time course analysis of cytoplasmic and nuclear pDM128RZ RNAs. Plasmids pDM128RZ and PyE were together transfected into COS7 cells, with cotransfection of pRSV-Rev. Nuclear and cytoplasmic RNAs were prepared 0, 60, and 120 min after treatment with a 5-μg/ml final concentration of actinomycin D and were assayed by quantitative RNase protection. For cytoplasmic RNA, three times more RNA samples were used in RNase protection and exposure was three times longer. The half-life times have been normalized to the levels of the internal control PyE spliced mRNAs (data not shown). Labels are the same as in Fig. 4.

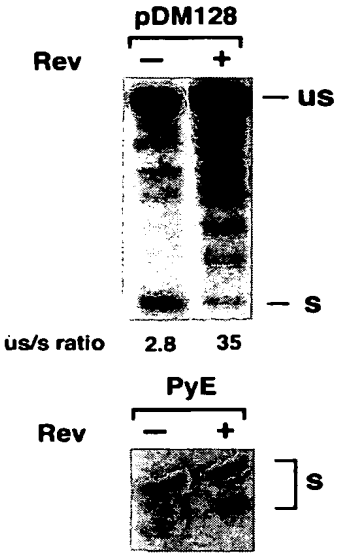

FIG. 7. Rev interferes with splicing. Plasmids pDM128 and PyE were transfected into COS7 cells, with or without cotransfection of pRSV-Rev. Total RNAs were extracted and assayed by quantitative RNase protection. Top panel, expression of pDM128. Bottom panel, expression of PyE. us, unspliced RNA; s, spliced RNA.

of rc RNA in the cytoplasm is not due to rapid degradation; rather, it is due to poor export. Unexpectedly, the half-life of nuclear rc RNA increased about threefold in the presence of Rev over that in the absence of Rev (compare Fig. 6, lanes 4 to 6, to Fig. 4A, lanes 4 to 6). This suggests that Rev stabilizes rc RNA in the nucleus. From the above results we suggest that polyadenylation is required for Rev to stimulate nuclear export of RRE-containing mRNAs.

A close inspection of the data shown in Fig. 3 and 5 revealed that an increased level of unspliced cytoplasmic RNA produced by pDM128 was coincident with a lower level of cytoplasmic spliced RNA, and that these changes were associated with the presence of Rev. This suggested the possibility that Rev may interfere with splicing. In fact, if the sum of cytoplasmic and nuclear signals was considered, this result was also evident (data not shown). These results were observed in more than three independent experiments. To determine directly whether Rev interferes with splicing, total cellular RNA derived from cells transfected with pDM128 and PyE, with or without cotransfection of the Rev expression vector, was subjected to RNase protection analysis (Fig. 7). The ratios between unspliced and spliced pDM128 mRNAs are 2.8 and 35 in the absence and presence of Rev, respectively. In addition, the steady state level of spliced pDM128 mRNA (after being normalized for the level of PyE spliced RNA) is about fourfold higher in the absence of Rev than that in the presence of Rev. These results indicate that fewer RNAs were spliced in the presence of Rev (see Discussion).

A long poly(A) stretch near the 3′ end of an RNA molecule is not sufficient for export. To determine whether a poly(A) sequence near the 3′ end of mRNAs is sufficient for nucleocytoplasmic transport, we replaced the poly(A) signal in pDM128 with the hammerhead ribozyme cassette and then inserted a 90-bp poly d(A/T) stretch immediately upstream of the histone stem-loop to build pDM128RZA (Fig. 1). To analyze the subcellular localization of RNAs, pDM128RZA and PyE were transfected into COS7 cells with or without cotransfection of pRSV-Rev. Results of RNase protection assays are presented in Fig. 8. The 818-nt riboprobe could only distinguish between spliced and unspliced RNAs and could not distinguish between ribozyme-cleaved and uncleaved RNA species (Fig. 1). The transport phenotype displayed by unspliced transcripts (including ribozyme-cleaved and uncleaved RNAs), in both the presence and absence of Rev, is strikingly similar to that of pDM128RZ (compared with Fig. 3 and 5). Actinomycin D chase experiments (data not shown) revealed that the poly(A) stretch stabilized pDM128RZA transcripts to

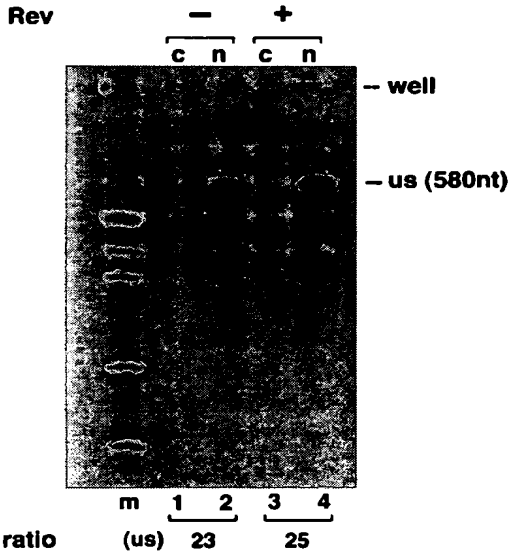

FIG. 8. A long poly(A) stretch is not sufficient for mRNA export. Plasmids pDM128RZA and PyE were transfected into COS7 cells, with or without cotransfection of pRSV-Rev. Nuclear and cytoplasmic RNAs were analyzed by RNase protection. us, unspliced pDM128RZA RNA; other labels are the same as in Fig. 3.

about the same extent as that seen by Rev on pDM128RZ transcripts (Fig. 6). In addition, Rev addition did not lead to further stabilization (data not shown). Taken together, these results suggest that a poly(A) stretch alone may not be sufficient for mRNA nuclear export. Rather, productive interaction of the pre-mRNA with the cellular polyadenylation machinery appears to be required. However, these results do not rule out the possibility that a poly(A) stretch at the very 3′ terminus of a transcript can stimulate export.

DISCUSSION

In this study we have employed a *cis*-acting hammerhead ribozyme to uncouple 3′-end formation of reporter gene transcripts from the cellular polyadenylation apparatus and have demonstrated that polyadenylation is required for nucleocytoplasmic transport of mRNA. The constructs used were derived from the HIV-1 genome, with the cytoplasmic localization of unspliced gene transcripts being regulated by Rev. In the absence of Rev, polyadenylated unspliced transcripts were readily detected in the cytoplasm of the transfected COS7 cells (Fig. 3, lanes 1 and 2). However, ribozyme-cleaved unspliced RNAs failed to appear in the cytoplasm. Instead, they accumulated to a high level in the nucleus (Fig. 3, lanes 3 and 4). Moreover, the intracellular distribution pattern of ribozyme-cleaved RNAs remained unchanged when Rev is present, whereas the level of cytoplasmic polyadenylated mRNAs was significantly increased (Fig. 5, compare lanes 1 and 2 with lanes 3 and 4). RNA stability measurements using actinomycin D suggested that the distinct distribution patterns of the two classes of transcripts within the cell were due to their differential nucleocytoplasmic transport. We therefore hypothesize that polyadenylation is required for mRNA nuclear export and this requirement cannot be bypassed by Rev/RRE. These findings are consistent with the observation that polyoma early region antisense RNAs produced by ribozyme cleavage are retained in the nucleus (29). However, these conclusions are inconsistent with results obtained from experiments which showed that in vitro synthesized, nonpolyadenylated mRNAs could be exported to the cytoplasm after microinjection into *Xenopus* oocyte nuclei (16, 18). Moreover, in one case an intron lariat containing the RRE sequence was found to be exported to the oocyte cytoplasm only in the presence of Rev (16). The discrepancies may be due to the different systems and techniques used. Alternatively, they may result from the different sizes of RNAs used (RNAs used for microinjection were less than 500 nt, while RNAs expressed from our vectors were around 4,000 nt). It is also possible that microinjected RNAs may enter an export pathway differently from endogenously expressed RNAs. Finally, it cannot be ruled out that microinjected RNAs underwent polyadenylation in vivo before being exported to the cytoplasm.

Polyadenylation is initiated by specific recognition of the highly conserved AAUAAA hexanucleotide sequence present on pre-mRNA substrates by polyadenylation factors, followed by endonucleolytic cleavage of the pre-mRNA 5 to 40 nt downstream of the hexanucleotide and the addition of 150 to 200 adenosine residues at the 3′ end (47). Some polyadenylated transcripts contain less than 100 adenosine residues (40). The poly(A) sequence itself has been implicated in regulating mRNA stability and translation in the cytoplasm (1, 22). The next question we asked, therefore, was whether a poly(A) stretch alone is sufficient for mRNA export. Results suggest that this is not the case and lead to the proposal that the process of polyadenylation is essential for mRNA export. The key to this hypothesis is that RNAs whose 3′ ends were formed by ribozyme cleavage and that possessed 90 adenosine residues near their 3′ ends failed to enter the cytoplasm, whether in the presence or in the absence of Rev (Fig. 8).

Poly(A) encoded in the above reporter gene differs from the normal poly(A) in that it has a 31-nt 3′ extension. One might argue that these non-A residues interfere with the cellular export machinery, or that the assembly of an export-competent complex requires a poly(A) stretch at the very 3′ end. Further experimentation is required to determine whether either of these models is true. However, it remains possible that binding of cellular proteins to the 90-nt-long A stretch is not itself sufficient for mRNA export, or that the binding of an important factor or factors which are essential for promoting mRNA export can occur only during the polyadenylation process. Further evidence supporting this comes from observations that a histone stem-loop structure alone was not sufficient for neomycin mRNA nuclear export; rather, the entire histone 3′ processing machinery was required for this event (10). Considering this with our results, we propose that the ability to link RNA 3′ processing and nucleocytoplasmic export of mRNAs may be a general, intrinsic property of 3′ processing signals. Finally, since it was shown that a 55- to 65-nt-long poly(A) stretch could promote mRNA export (25), we suggest that the 90-nt poly(A) stretch used here should also be sufficiently long for export.

cis elements present in our reporter transcripts are not responsible for the nuclear retention of these RNAs. First, the 2′,3′ cyclic phosphate terminus generated by ribozyme cleavage does not retain RNAs within the nucleus. This is supported by the observation that transcripts containing the histone coding region and a histone 3′ stem-loop generated by ribozyme cleavage were efficiently exported to the cytoplasm (10). Second, as normally processed histone mRNA can be efficiently transported to the cytoplasm, the same histone stem-loop present at the 3′ ends of our transcripts likewise cannot prevent the export of these RNAs. Finally, since unused 5′ and 3′ splice sites present in polyadenylated pDM128 transcripts do not retain RNAs in the nucleus in the presence of Rev, it is highly unlikely that the same splice sites retain ribozyme cleaved RNAs in the nucleus when Rev is present.

Another interesting finding of this study was that polyadenylation appeared to stabilize unspliced mRNA in the nucleus, although other differences in RNA structure might also have influenced nuclear lifetimes. Nuclear polyadenylated mRNAs were more stable than ribozyme-cleaved RNAs in the absence of Rev (Fig. 4). Rev was also found to enhance the stability of ribozyme-cleaved unspliced RNAs in the nucleus (compare Fig. 4 with Fig. 6). Whether these stabilization effects are manifestations of the same mechanism is unknown. It has been shown that the function of Rev is dosage dependent and requires Rev oligomerization (24, 35, 39, 48; unpublished results). We suggest, therefore, that binding of polyadenylation factors and/or poly(A)-binding proteins to the poly(A) sequence, binding of Rev to the RRE, and more importantly, productive protein-protein interactions between these proteins and other cellular factors, efficiently protect the substrate mRNAs from being degraded in the nucleus and thus ensure their availability for nuclear export.

Failure to see splicing in ribozyme-cleaved transcripts (Fig. 2, 3, 5, and 8) was quite surprising and interesting and may be the consequence of interference with "exon definition" during splice site selection and commitment (44). Terminal exon splicing may require recognition of both the poly(A) signal and the upstream 3′ splice site (31, 38, 44). In this model, a ribozyme would likely not be an acceptable "exon terminator"; therefore, terminal exon splicing could not occur.

Our studies also provide valuable insight into Rev function. In the presence of Rev, the steady-state level of the spliced mRNAs decreased compared with that in the absence of Rev, and this change was coincident with the increased expression of unspliced mRNAs (Fig. 3, 5, and 7). This indicates that Rev interferes with splicing. It is possible that this interference with splicing results from direct interaction of Rev with the cellular splicing machinery to free the unspliced mRNAs for nuclear export. Alternatively, it is possible that Rev binds to polyadenylated unspliced mRNAs and directly aids their rapid nuclear export. Export may be so rapid as to preclude access to the splicing machinery. Recent studies favor the second model, although the two are not necessarily mutually exclusive. In T lymphocytes, cytoplasmic levels of fully spliced mRNAs were independent of the presence of Rev (33). Moreover, Rev may shuttle between the nucleus and cytoplasm and direct RRE-containing mRNAs to the cytoplasm through its C-terminal activation domain (15, 23, 32, 37, 42). Finally, several nuclear proteins (including nuclear pore proteins) that interact with Rev have recently been identified (3, 17, 46), indicating that Rev may interact directly with the cellular export machinery. However, the reporter transcripts used in most of the above experiments were all polyadenylated (except in the case of *Xenopus* oocyte microinjection experiments [15]). Therefore, direct action of Rev in mRNA export does not exclude that polyadenylation is required for its function. Data from the microinjection experiments of Fischer et al. (15, 16) do not agree with our findings; possible explanations for these discrepancies have been discussed above.

Taking together the data we have presented here, one can envisage a model for the central importance of polyadenylation in general mRNA nuclear export. Polyadenylation may allow mRNAs to enter a pathway that leads to their efficient export. It is possible that only those that have undergone polyadenylation can be properly recognized by the nuclear pore complex and become accessible to the cellular export machinery. With regard to Rev's facilitation of mRNA export, binding of Rev to substrate mRNAs and its productive recruitment of cellular factors involved in export may occur only when mRNAs have been delivered to the appropriate intranuclear location by the action of the cellular polyadenylation machinery.

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EXHIBIT J

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MINIREVIEW

When Two Strands Are Better Than One: The Mediators and Modulators of the Cellular Responses to Double-Stranded RNA

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Double-stranded RNA is a potent inducer of interferon, a modulator of the expression of a number of other genes involved in the response of cells to virus infection, an activator of the interferon-induced antiviral state, and may be involved in differentiation, induction of apoptosis, and control of oncogenic transformation. This review will attempt to summarize what is known about the cellular proteins that act to mediate the response of cells to double-stranded RNA and the viral and cellular macromolecules that may be able to modulate these responses. © 1996 Academic Press, Inc.

THE PLAYERS

Most viruses induce the synthesis of dsRNA[2] at some time during their replication cycle. The presence of viral dsRNA appears to trigger many of the cellular responses to virus-infection, probably through activation of dsRNA-dependent enzymes, including the interferon-inducible protein kinase, PKR, and the interferon-inducible enzyme, 2′,5′ oligoadenylate synthetase. As countermeasures, many viruses have evolved mechanisms to mask the effects of dsRNA on cells. This review will first summarize what is known about synthesis of dsRNA in virus-infected (and perhaps uninfected) cells, will then tackle the dsRNA-activatable enzymes that are present in cells, and finally will review the viral and cellular products that can modulate the cellular response to dsRNA.

SOURCES OF dsRNA

Before discussing the macromolecules in cells that mediate the response to dsRNA it is important to get a glimpse of the sources of RNA that can interact with these molecules in infected and perhaps uninfected cells. Actually identifying the potential sources of dsRNA in infected cells has in fact been problematic over the years. Minute quantities of dsRNA, as little as one molecule per cell (Marcus and Sekellick, 1977), can have profound effects on cellular physiology. It is difficult to detect such miniscule quantities of preexisting dsRNA in cell extracts. This problem is exacerbated by the potential reassociation of complementary strands of RNA during extract preparation, especially during extraction with phenol (Kohne *et al.*, 1977), always calling into question whether any isolated dsRNA preexisted in the cell or was in fact an artifact of reassociation during isolation. Nonetheless, binding of anti-dsRNA antibodies to viroplasm of extracted whole cells (Lee *et al.*, 1994a) and isolation of viral mutants that alter activation of dsRNA-dependent processes (Kitajewski *et al.*, 1986; Beattie *et al.*, 1991, 1995a,b; Chang *et al.*, 1995) all suggest that dsRNA does in fact exist within virus-infected cells.

For viruses with dsRNA genomes the obvious suspect is the genome itself. However, completely uncoated dsRNA genome has not been detected in cells infected with dsRNA-viruses (Schiff and Fields, 1990). For the reoviruses, input viral dsRNA remains within the inner capsid throughout the viral life cycle, and progeny genome is only synthesized after assembly of positive sense single-stranded progeny RNA into subviral particles. It is likely that the machinations that dsRNA viruses go through to prevent exposure of naked dsRNA in cells is a consequence of the profound effects that dsRNA has on the physiology of the cell. Nonetheless, it could be that minute amounts of incorrectly uncoated or packaged genome might be present in infected cells and could activate the known dsRNA-dependent enzymes. Alterna-

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[2] Abbreviations used: DRAD, dsRNA adenosine deaminase; dsRNA, double-stranded RNA; E3L, the vaccinia virus gene coding for a dsRNA-binding protein inhibitor of PKR; EBER, Epstein–Barr virus-encoded small RNA; eIF-2, eukaryotic protein synthesis initiation factor 2; K3L, the vaccinia virus gene coding for an eIF-2α homologue inhibitor of PKR; PKR, the interferon-inducible, dsRNA-dependent protein kinase; RRE, the HIV rev responsive element; tar, the HIV tat responsive element; tat, the HIV transactivator of gene expression; VAI RNA, adenovirus-encoded small RNA I; VAII RNA, adenovirus-encoded small RNA II.

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tively, secondary structure on mRNA might be involved in activating these enzymes in infected cells. Such structure has been implicated in the poor translation of the reovirus s1 mRNA (Henry *et al.*, 1994).

For ssRNA viruses the obvious culprit is replicative intermediate in infected cells. This is supported by the isolation of both positive and negative sense RNA bound to the dsRNA-binding protein 2′,5′ oligoadenylate synthetase from EMCV-infected cells (Gribaudo *et al.*, 1991) and by binding of antisera that recognizes dsRNA to viroplasm in rubella and semliki forest virus-infected cells (Lee *et al.*, 1994a). Viral dsRNA isolated from influenza virus-infected lungs was able to induce both the local and systemic cytotoxic effects typical of influenza virus-infection when injected into experimental animals (Majde *et al.*, 1991; Kimura *et al.*, 1992). Again, however, it is unclear how much true dsRNA, i.e., full duplexes between positive and negative sense RNA, exists in cells infected with ssRNA viruses.

For DNA viruses dsRNA appears to accumulate as the result of overlapping convergent transcription. At late times after infection with vaccinia virus, viral transcripts fail to terminate at discrete sites at the ends of genes (Moss, 1990). Thus, complementary mRNAs are produced from genes transcribed in opposing directions (Colby and Duesberg, 1969; Duesberg and Colby, 1969; Colby *et al.*, 1971; Boone *et al.*, 1979). The vaccinia virus A18R gene product modulates transcription termination, thereby altering the level of dsRNA that accumulates in infected cells, with defects in A18R leading to excess dsRNA (Bayliss and Condit, 1993; Simpson and Condit, 1994). Similar complementary transcripts have been detected in adenovirus- (Pettersson and Philipson, 1974; Maran and Mathews, 1988), herpes simplex virus- (Jaquemont and Roizman, 1975), and SV-40- (Aloni, 1972) infected cells. Again, the actual extent of hybridization between these complementary strands in infected cells is unclear, although a large fraction of the RNA in vaccinia virus-infected cells is ribonuclease resistant even before deproteination (Colby *et al.*, 1971). In addition, activation of the dsRNA-dependent pathways has been detected in cells infected with either adenovirus (Kitajewski *et al.*, 1986) or vaccinia virus (Beattie *et al.*, 1991, 1995a,b; Chang *et al.*, 1995) deleted for inhibitors of these pathways.

While fully duplexed RNA has not been detected in retrovirus-infected cells, the HIV and HTLV genomes contain large domains of secondary structure (*tar* and *RRE/RxRE*) that function to regulate gene expression and can interact with several dsRNA-binding proteins (Sengupta and Silverman, 1989; Edery *et al.*, 1989; Schroder *et al.*, 1990; Silverman and Sengupta, 1990; Gunnery *et al.*, 1990, 1992; Gatignol *et al.*, 1993; Park *et al.*, 1994; Maitra *et al.*, 1994).

Perhaps some of the most intriguing findings have been the detection of dsRNA-like molecules in apparently uninfected cells (Maran and Mathews, 1988; Li and Petryshyn, 1991), which are capable of activating the dsRNA-dependent protein kinase, PKR, *in vitro*. This RNA is apparently poly(A) rich and may contain topologically unlinked complementary strands. Again, it is unclear if these hybrids exist in cells or hybridize during RNA isolation, although PKR activation has been detected in differentiating adipocytes (Li and Petryshyn, 1991), from which dsRNA can be isolated, and after IL-3 deprivation of an IL-3-dependent murine cell line (Ito *et al.*, 1994).

MEDIATORS OF THE CELLULAR RESPONSE TO dsRNA

Two of the cellular gene products whose activities are most clearly regulated by dsRNA are the protein kinase, PKR, and the enzyme 2′,5′ oligoadenylate synthetase (Sen and Lengyel, 1992; Pestka *et al.*, 1987). Both enzymes can be induced in cells treated with interferon, and both enzymes can bind to and be potently activated at a posttranslational step by dsRNA. In the case of PKR, activation occurs concomitantly with intermolecular (Kostura and Mathews 1989; Thomis and Samuel 1995) and perhaps intramolecular (Berry *et al.*, 1985; Galabru *et al.*, 1989) phosphorylation, which may be accompanied by dimerization (Langland and Jacobs, 1992; Patel *et al.*, 1995). The level of PKR activation may be regulated in cells by depletion of Ca^{+2} stores in the endoplasmic reticulum (Prostko *et al.*, 1995). Once activated, PKR can phosphorylate a number of exogenous substrates including the small (α) subunit of the protein synthesis initiation factor eIF-2 (Farrell *et al.*, 1977; Levin and London, 1978; Samuel, 1979), the NFκB inhibitor IκB (Maran *et al.*, 1994; Kumar *et al.*, 1994; Offermann *et al.*, 1995), and histone proteins (Jacobs and Imani, 1988; Galabru and Hovanessian, 1985). eIF-2α phosphorylation can lead to an inhibition of the initiation of protein synthesis. eIF-2α phosphorylation by PKR is presumed to be involved in the interferon-mediated inhibition of replication of a number of viruses. Constitutive expression of either human (Meurs *et al.*, 1992) or mouse (Baier *et al.* 1993) PKR leads to an inhibition of replication of EMCV, but not VSV. For both adenovirus (Kitajewski *et al.* 1986) and vaccinia virus (Beattie *et al.*, 1991, 1995a,b; Chang *et al.*, 1995), deletion of inhibitors of PKR (VAI RNA for adenovirus and the E3L or K3L genes for vaccinia virus) leads to increased phosphorylation of eIF-2α and renders these normally interferon-resistant viruses sensitive to the effects of interferon. In the case of adenovirus, deletion of the VAI gene can be complemented by overexpression of a nonphosphorylatable variant of eIF-2α (Davies *et al.*, 1989). Phosphorylation of IκB by PKR can lead to IκB degradation and subsequent activation of NFκB (Maran *et al.*, 1994; Kumar *et al.*, 1994; Offermann *et al.*, 1995), perhaps through a reactive oxygen-mediated pathway (Schreck *et al.*, 1991). The activation of NFκB mediated

by dsRNA may be involved in induction of interferon-β gene expression and of the other cellular genes whose transcription is influenced by dsRNA. PKR-mediated phosphorylation of histone proteins has only been detected *in vitro* and its biological significance is at present unclear. Activated PKR may also be able to induce apoptosis in vaccinia virus-infected cells (Lee and Esteban, 1994), although neither the activators nor substrates involved in this response have been characterized. Inhibition of endogenous PKR, either by expression of dominant negative mutants of PKR (Koromilas *et al.*, 1992; Meurs *et al.*, 1993) or by expression of a natural cellular inhibitor of PKR (Barber *et al.*, 1994) produced a transformed phenotype in cells, as did overexpression of a nonphosphorylatable mutant of eIF-2α (Donze *et al.*, 1995). PKR has been found in most mammalian cells analyzed. An analogous enzyme, which is immunologically cross-reactive with human PKR, has been identified in plant cells (Langland *et al.*, 1995) and is inducible by virus and viroid infection (Crum *et al.*, 1988; Hiddinga *et al.*, 1988; Roth and He, 1994).

Activation of 2′,5′ oligoadenylate synthetase by dsRNA is likely not a consequence of a posttranslational modification, but of a conformational change in the enzyme induced by binding to dsRNA. Once activated, the enzyme can polymerize ATP and other nucleotides in novel 2′,5′ linkages (Kerr and Brown, 1978). These 2′,5′ oligoadenylates can activate a ribonuclease, RNase L, that can cleave ssRNAs, including rRNA, at UpA, UpG, or UpU residues (Silverman *et al.*, 1988; Bisbal *et al.*, 1989; Baglioni *et al.*, 1979; Nilsen *et al.*, 1982; Schroder *et al.*, 1989; Floyd-Smith *et al.*, 1981; Wreschner *et al.*, 1981). Several isoforms of 2′,5′ oligoadenylate synthetase have been identified, and cDNA clones for two of the isoforms have been characterized (Laurent *et al.*, 1983; Yang *et al.*, 1981; Chebath *et al.*, 1987a; Saunders *et al.*, 1985; Ilson *et al.*, 1986; Rosenblum *et al.*, 1988; Marie *et al.*, 1990; Wathelet *et al.*, 1986). The smaller of the two isoforms of 2′,5′ oligoadenylate synthetase appears to be sufficient to inhibit replication of EMCV and vaccinia virus but not VSV in cells transfected with plasmid engineered to constitutively express the enzyme (Chebath *et al.*, 1987b; Grun *et al.*, 1987). In addition, expression of antisense RNA to the small isoform of 2′,5′ oligoadenylate synthetase prevented the interferon-mediated inhibition of EMCV replication (Benedetti *et al.*, 1987), as did expression of a dominant negative mutant of RNase L (Hassel *et al.*, 1993). Both negative and positive sense picornaviral RNA have been found bound to 2′,5′ oligoadenylate synthetase in EMCV-infected cells (Gribaudo *et al.*, 1991), consistent with activation of the pathway in picornavirus-infected cells.

Both PKR and 2′,5′ oligoadenylate synthetase bind specifically to dsRNA or RNA with secondary structure, including the reovirus s1 mRNA (Bischoff and Samuel, 1989), adenovirus VAI RNA (Desai *et al.*, 1995; Mathews and Shenk, 1991), and the *tar* region of the HIV-1 RNA (Maitra *et al.*, 1994; Silverman and Sengupta, 1990; Gunnery *et al.*, 1990, 1992; Edery *et al.*, 1989; Sengupta and Silverman, 1989; Schroder *et al.*, 1990). PKR binds to RNA with K_ds in the nM range (McCormack and Samuel, 1995; Schmedt *et al.*, 1995). For PKR, the amino-terminal third of the protein appears to be both necessary and sufficient for binding to dsRNA (McCormack *et al.*, 1992; Patel and Sen, 1992; Feng *et al.*, 1992; Katze *et al.*, 1991). This region contains two copies of a motif that has been found in a number of proteins that bind specifically to dsRNA or structured RNA (St. Johnston *et al.*, 1993; Chang *et al.*, 1992; Chang and Jacobs, 1993; McCormack *et al.*, 1992). Both copies of the motif appear necessary for high affinity binding of PKR to dsRNA, although the amino-proximal motif seems to be more important for binding and activity than the internal motif. Mutation of several residues in PKR, conserved among most proteins containing the motif, interfered with binding to dsRNA (McCormack *et al.*, 1994; Green *et al.*, 1995; Romano *et al.*, 1995; McMillan *et al.*, 1995; Clarke and Mathews, 1995).

The dsRNA-binding domain for the 2′,5′ oligoadenylate synthetases has not been as well defined as the domains on PKR. Sequences within the amino-terminal 158 residues on the small (42-kDa) isoform of 2′,5′ oligoadenylate synthetase are necessary for binding to dsRNA (Ghosh *et al.*, 1991). This region of 2′,5′ oligoadenylate synthetase contains no sequences homologous to any other proteins in the database. The cDNA clone of the larger, 69-kDa isoform of 2′,5′ oligoadenylate synthetase contains a duplication of sequences homologous to the small isoform of 2′,5′ oligoadenylate synthetase (Marie and Hovanessian, 1992). As of yet the sequences necessary for the 69-kDa isoform to bind to dsRNA have not been identified.

Both PKR and the various forms of 2′,5′ oligoadenylate synthetase have been found in the cytoplasm as well as the nucleus (Jimenez Garcia *et al.*, 1993; Jeffrey *et al.*, 1995; Saunders *et al.*, 1985; Rosenblum *et al.*, 1988) of cells. For PKR approximately 80% of the enzyme is found in the cytoplasm while 20% is found in the nucleus (Jeffrey *et al.*, 1995). The nuclear form of the enzyme was concentrated in nucleoli and was apparently relatively underphosphorylated, compared to cytoplasmic enzyme. The cytoplasmic form of PKR is both associated with ribosomes (80%) and free in the cytoplasm (20%). Cytoplasmic PKR not bound to ribosomes is partially phosphorylated and a dimer (Langland and Jacobs, 1992). The different roles that these differentially localized and differentially phosphorylated forms of the kinase play in inhibition of virus replication or in normal cell physiology is at present unclear.

VIRAL MODULATORS OF THE RESPONSE TO dsRNA

Given the efficiency of the dsRNA-dependent enzymes at inhibiting virus infection, it is perhaps not surprising

that a number of viruses have evolved pathways to counteract activation and/or activity of both 2′,5′ oligoadenylate synthetase and PKR. The most well-characterized PKR inhibitor is the adenovirus VAI RNA (Mathews and Shenk, 1991). VAI RNA is found in abundant amounts at late times after infection with adenovirus. A second adenovirus-encoded small RNA, VAII RNA, is synthesized in lower amounts in infected cells. Virus deleted for VAI replicates poorly, while virus deleted for VAII replicates as well as wild-type virus (Thimmappaya *et al.*, 1982). VAI RNA is highly structured in solution (Furtado *et al.*, 1989; Mellits and Mathews, 1988) and binds to PKR in competition with dsRNA (Galabru *et al.*, 1989; Katze *et al.*, 1987; Kostura and Mathews, 1989; Mellits *et al.*, 1990) but fails to efficiently lead to PKR autophosphorylation or activation. The ability to bind to PKR in competition with dsRNA is not sufficient for VAI function, since several mutants of VAI that bind PKR *in vitro* do not effectively support adenovirus replication (Mellits *et al.*, 1990). Binding to PKR requires an apical stem structure of at least half a turn in length (Clarke *et al.*, 1994), but a central domain of complex structure is required to inhibit PKR activation (Pe'ery *et al.*, 1993; Ghadge *et al.*, 1994). Deletion of the VAI gene leads to a virus that replicates poorly and is sensitive to the antiviral effects of interferon (Thimmappaya *et al.*, 1982; Kitajewski *et al.*, 1986). Analogous Epstein–Barr virus-encoded structured small RNAs (EBERs) can inhibit activation of PKR (Clarke *et al.*, 1991; Sharp *et al.*, 1993; Clemens *et al.*, 1994). The Epstein–Barr virus encoded small RNAs can at least partially complement adenovirus deleted for VAI RNA (Bhat and Thimmappaya, 1983) as can SV-40 large T antigen (Rajan *et al.*, 1995). However, the role of at least the Epstein–Barr virus encoded small RNAs in infected cells is unclear since deletion of the gene for these RNAs has no effect on sensitivity of the virus to interferon treatment (Swaminathan *et al.*, 1992). EBERs have been shown to bind to the ribosomal protein L22 (Toczyski *et al.*, 1994). Again, the functional significance of binding to L22 is at present unclear. VAI RNA can interact with 2′,5′ oligoadenylate synthetase, but activates rather than inhibits the enzyme (Desai *et al.*, 1995). As is true of many of the macromolecules described in this review, VAI RNA could be detected in the nucleus as well as the cytoplasm of infected and transfected cells (Jimenez Garcia *et al.*, 1993). The role that nuclear localization of VAI RNA plays in virus replication is at present unclear.

Vaccinia virus-infected cells contain at least two products capable of inhibiting PKR. The protein product of the K3L gene has partial homology to one of the substrates of PKR, eIF-2α (Beattie *et al.*, 1991; Goebel *et al.*, 1990), and can inhibit phosphorylation of this protein synthesis initiation factor (Carroll *et al.*, 1993; Davies *et al.*, 1992, 1993; Jagus and Gray, 1994). The K3L gene product is thought to function by binding to PKR competitively with eIF-2 (Carroll *et al.*, 1993; Jagus and Gray, 1994). Virus deleted for K3L is interferon sensitive (Beattie *et al.*, 1991), but does not have a host range different from wild-type vaccinia virus (see below) and does not induce apoptosis in infected cells (Lee and Esteban, 1994).

The vaccinia virus E3L gene codes for a second modulator of dsRNA in virus-infected cells. The E3L gene codes for two proteins that can both bind specifically to dsRNA (Watson and Jacobs, 1991; Chang *et al.*, 1992; Yuwen *et al.*, 1993). Cloned E3L gene products can inhibit activation of PKR (Chang *et al.*, 1992). Inhibition *in vitro* appears not to be catalytic (Whitaker-Dowling and Youngner, 1983; Jagus and Gray, 1994) and can be overcome with increasing concentrations of dsRNA (Whitaker-Dowling and Youngner, 1983; Watson and Jacobs, 1991). Deletion of the E3L gene from vaccinia virus leads to loss of kinase inhibitory activity and to degradation of rRNA characteristic of activation of the 2′,5′ oligoadenylate synthetase/RNase L pathway (Beattie *et al.*, 1995a). Virus deleted for E3L has a host-range phenotype, in that it will replicate in RK-13 and CEF cells but not in HeLa or Vero cells (Beattie *et al.*, 1995a,b; Chang *et al.*, 1995). Replication in L cells is semipermissive (Beattie *et al.*, 1995). In those cells in which virus deleted for E3L does replicate, replication is sensitive to the anti-viral effects of interferon (wild-type vaccinia virus is resistant to the effects of interferon in most cell types tested) (Beattie *et al.*, 1995a; Chang *et al.*, 1995). Virus deleted for E3L induces apoptosis in HeLa cells (Lee and Esteban, 1994). Thus, the host-range phenotype may be a consequence of induction of apoptosis in cells. Induction of apoptosis by virus deleted for E3L, together with the fact that PKR expressed from wild-type vaccinia virus induces apoptosis (Lee and Esteban, 1994), suggests that viral dsRNA may be inducing apoptosis by activating PKR. Results on influenza virus-mediated induction of apoptosis suggest that dsRNA might mediate its effects by induction of fas antigen, again, perhaps through activation of PKR (Takizawa *et al.*, 1995). Several other proteins that can bind to dsRNA can substitute for E3L in allowing replication in HeLa cells (Park *et al.*, 1994; Langland *et al.*, 1994; Beattie *et al.*, 1995a). Interestingly, the E3L gene products are detected primarily in the nucleus at early times after infection and in transfected cells (Yuwen *et al.*, 1993). The role that migration of these gene products to the nucleus plays in virus replication is at present unclear, although a mutant of E3L encoding a protein that fails to migrate to the nucleus could rescue the host-range defect of vaccinia virus deleted for E3L (Chang *et al.*, 1995).

The vaccinia virus E3L gene contains a single copy of the dsRNA-binding motif also found in PKR (Chang *et al.*, 1992). Deletion and point mutation analysis indicates that this motif is necessary for binding of the E3L gene products to dsRNA (Chang and Jacobs, 1993). The ability of mutants of E3L to support replication of vaccinia virus in HeLa cells correlates with their ability to bind dsRNA

(Chang *et al.*, 1995). A similar motif is found in the porcine group C rotavirus NSP3 gene (Langland *et al.*, 1994). While a direct mutational analysis of NSP3 gene function is not possible due to the lack of a gene replacement system in rotaviruses, the NSP3 gene can fully complement deletion of the E3L gene in vaccinia virus. The NSP3 gene is predicted to encode a 45-kDa protein. However, when NSP3 is expressed either *in vitro*, or in transfected cells, the 45-kDa product is cleaved into 38- and 8-kDa proteins. The 8-kDa protein contains the dsRNA-binding motif and binds to dsRNA. This 8-kDa polypeptide has been detected in cells infected with group C rotaviruses (Langland *et al.*, 1994), and thus is the smallest known natural protein that can specifically bind dsRNA and antagonize at least some of the effects of dsRNA.

The reovirus $\sigma 3$ protein can also bind specifically to dsRNA (Huismans and Joklik, 1976), even though it has, at best, limited homology to other known dsRNA-binding proteins. $\sigma 3$ can inhibit PKR activation *in vitro* (Imani and Jacobs, 1988) and in transfected cells (Giantini and Shatkin, 1989; Lloyd and Shatkin, 1992; Denzler and Jacobs, 1994). The gene encoding $\sigma 3$ (S4) can also partially complement deletion of E3L from vaccinia virus (Beattie *et al.*, 1995a) and deletion of VAI from adenovirus (Lloyd and Shatkin, 1992). Some strains of reovirus are relatively resistant to the effects of interferon while other strains are quite sensitive (Jacobs and Ferguson, 1991). As of yet the gene encoding resistance of reovirus to interferon has not been identified. Strains of reovirus also differ in their ability to inhibit translation of host mRNAs. The ability of reovirus to inhibit host protein synthesis maps to the gene encoding $\sigma 3$ (Sharpe and Fields, 1982). While all strains of mammalian reovirus thus far analyzed code for a $\sigma 3$ protein capable of binding dsRNA (Seliger *et al.*, 1992), there may be strain differences in the affinity of $\sigma 3$ for dsRNA, in the amount of $\sigma 3$ made in infected cells, or in the ability of $\sigma 3$ to bind to another viral protein, $\mu 1$, which can abrogate binding of $\sigma 3$ to dsRNA. Binding to dsRNA has been mapped to a basic region in the carboxy-half of the protein (Schiff *et al.*, 1988; Miller and Samuel, 1992; Denzler and Jacobs, 1994; Mabrouk *et al.*, 1995). Mutations of the protein in this domain that inhibit dsRNA-binding fail to support replication of vaccinia virus deleted for E3L in HeLa cells (Beattie *et al.*, 1995a).

In addition to the specific dsRNA-binding noted for the E3L gene products and $\sigma 3$, several viral proteins appear to bind to several different nucleic acids, including dsRNA. The influenza virus NS-1 protein can bind to both negative sense influenza virus RNA (Hatada *et al.*, 1992) and dsRNA (Hatada and Fukuda, 1992). NS-1 protein can act as an inhibitor *in vitro* of PKR and can block the dsRNA-mediated inhibition of translation *in vitro* (Qian *et al.*, 1995; Lu *et al.*, 1995). In addition, mutants of NS-1 have been shown to alter translation of viral RNAs in infected cells, and NS-1 expressed in HeLa cells can stimulate translation of reporter mRNAs containing 5′ untranslated regions of influenza virus mRNAs (Enami *et al.*, 1994). The hantavirus core protein appears also to bind to ssRNA and dsRNA (Gott *et al.*, 1993), while the reovirus $\lambda 1$ protein can bind to either dsRNA or dsDNA (Lemay and Danis, 1994).

HIV-infected cells contain a number of macromolecules capable of modulating the activity of PKR. As indicated previously, the *tar* stem–loop structure at the 5′-end of HIV genome RNA and mRNA can bind to PKR (Maitra *et al.*, 1994; Silverman and Sengupta, 1990; Gunnery *et al.*, 1990, 1992; Edery *et al.*, 1989). PKR binds to *tar* with 100-fold lower affinity than to either fully duplexed RNA or VAI RNA (McCormack and Samuel, 1995). Alternative investigators have argued that *tar* RNA sequences can either activate (Maitra *et al.*, 1994; Edery *et al.*, 1989) or antagonize (Gunnery *et al.*, 1992) activation of PKR. Interaction of PKR with *tar* could be inhibited by tat protein (Judware *et al.*, 1993).

CELLULAR MODULATORS OF THE RESPONSE TO dsRNA

Uninfected cells also contain a number of factors capable of modifying the effects of dsRNA. Human, bovine, mouse, and monkey cells contain a latent inhibitor of PKR, termed P58 (Lee *et al.*, 1990, 1992, 1994b; Lee and Katze, 1994; Barber *et al.*, 1994). The latent inhibitor could be separated from an "anti-inhibitor" either by precipitation with ammonium sulfate or by infection with influenza virus (Lee *et al.*, 1990). Active inhibitor decreases both PKR activation and activity toward eIF-2α, in an unknown manner (Lee *et al.*, 1990). The gene for P58 has been cloned, sequenced, and expressed. The protein is a member of a family of proteins, called the tetratricopeptide family, which includes cdc23, cdc16, and bimA, that may be involved in regulation of the cell cycle (Barber *et al.*, 1994; Lee *et al.*, 1994b). Overexpression of P58 results in transformation of cells in culture, presumably by inhibiting endogenous PKR (Barber *et al.*, 1994; Lee *et al.*, 1994b).

Uninfected cells also contain an eIF-2 associated protein, called p67 (Datta *et al.*, 1989) that can block PKR-mediated phosphorylation of either eIF-2α or histone proteins (Ray *et al.*, 1992). p67 has also been reported to block autophosphorylation of PKR (Ray *et al.*, 1992). p67 may be a general inhibitor of eIF-2α phosphorylation in that it can prevent phosphorylation mediated by the heme regulated eIF-2α kinase (Ray *et al.*, 1993). The inhibitor is degraded in serum-starved cells and its synthesis is induced by subsequent mitogen treatment (Ray *et al.*, 1992). Activity of the inhibitor may also be regulated by deglycosylation (Datta *et al.*, 1989).

Undifferentiated preadipocytes contain a 15-kDa protein inhibitor of PKR (Judware and Petryshyn, 1991, 1992). The inhibitor appears to block interaction of PKR

with dsRNA, but does not interact with dsRNA activator (Judware and Petryshyn, 1992). It has been suggested that the inhibitor binds directly to PKR to block its interaction with dsRNA.

The human cellular protein, TRBP (Gatignol *et al.*, 1991), has been shown to bind to dsRNA (Gatignol *et al.*, 1993; Park *et al.*, 1994) and to inhibit activation of PKR *in vitro* (Park *et al.*, 1994). TRBP contains three copies of the dsRNA-binding motif (Gatignol *et al.*, 1993; Park *et al.*, 1994). Overexpression of TRBP can complement the host-range defect of vaccinia virus deleted for the E3L gene (Park *et al.*, 1994). The role of TRBP in uninfected and in virus-infected cells is at present unclear, although human TRBP can bind to *tar* and RRE sequences on HIV RNA (Gatignol *et al.*, 1991, 1993; Park *et al.*, 1994) and can coimmunoprecipitate HIV RNA from infected cells (Gatignol *et al.*, 1993). A similar protein has been identified in *Xenopus levis* cells (St. Johnston *et al.*, 1993).

An adenosine deaminase that uses dsRNA as a substrate (dsRNA adenosine deaminase, DRADA) has been identified in a number of cells (O'Connell and Keller, 1994; Kim *et al.*, 1994; Hough and Bass, 1994; Morrissey and Kirkegaard, 1991; Nishikura, 1992). The inosine formed by deamination of adenosine base pairs with cytosine rather than uridine, with two consequences. First, adenosine deamination destabilizes the RNA:RNA duplexes and, second, transcription of deaminated strands leads to insertion of cytosine residues rather than uridine residues and thus to hypermutation. This enzyme likely plays a role in RNA editing (Kim and Nishikura, 1993) and is likely responsible for hypermutation of certain viruses (Cattaneo, 1994), including measles virus isolated from patients with SSPE (Cattaneo and Billeter, 1992). Secondary structure in the *tar* region of the HIV-1 RNA is also a substrate for *Xenopus* DRADA (Sharmeen *et al.*, 1991), although it is unclear if HIV RNA interacts with this enzyme in infected cells.

Double-stranded RNA also seems likely to be involved in signal transduction mediated by the ras oncogene. v-Ras transformation of cells induces an inhibitor of PKR (Mundschau and Faller, 1992). The inhibitor is heat and organic solvent sensitive, suggesting that it contains a protein as a necessary component (Mundschau and Faller, 1992). The inhibitor migrates through gel filtration chromatography with a M_r of approximately 100,000 (Mundschau and Faller, 1994). The inhibitor acted *in trans* to prevent phosphorylation of PKR, through an as yet unknown mechanism. The v-ras-induced inhibitor of PKR may interfere with PDGF induction of immediate early response genes, suggesting that PDGF may function through activation of PKR (Mundschau and Faller, 1995).

Finally, *La* antigen can bind dsRNA (Xiao *et al.*, 1994) and EBER RNAs (Lerner *et al.*, 1981), as well as snRNAs, can unwind dsRNA, and can inhibit activation of PKR *In vitro* (Xiao *et al.*, 1994). Histone proteins, in addition to being substrates for PKR, bind to dsRNA as well as to dsDNA and can prevent activation of PKR *in vitro* (Jacobs and Imani, 1988). Given the recent identification of PKR in the nucleus of cells these proteins may be acting as inhibitors and/or substrates of PKR in cells.

CONCLUDING REMARKS

The role of dsRNA in the response of cells to virus infection has been evident for a number of years. Our increased understanding of the resources that viruses invest to protect themselves from this response adds credence to the critical role of dsRNA in the cells recognition and response to virus infection. The potential role of dsRNA in uninfected cells is just beginning to be gleaned. The future will no doubt see great progress in our understanding of the interaction between cellular RNAs and the dsRNA-activated machinery in cells, progress that will almost certainly utilize the reagents that viruses have given to us as probes.

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EXHIBIT 2

SUPPLEMENTAL AMENDMENT TO MAY 4, 2009 AMENDMENT
FILED IN RESPONSE TO NOVEMBER 3, 2008 OFFICE ACTION

Submitted: October 7, 2009

Serial No. 10/821,726

Filed: April 8, 2004

Applicants: Michael Wayne Graham et al.

Court of Appeals, Federal Circuit

In re O'Farrell

No. 87-1486

Decided August 10, 1988

PATENTS

1. Patentability/Validity — Obviousness — Evidence of (§115.0906)

Applicants' method of producing predetermined protein in stable form in host species of bacteria through genetic engineering is obvious within meaning of 35 USC 103 since reference, authored by two of three patent applicants and published more than one year prior to patent application date, contained detailed enabling methodology for practicing claimed invention, suggestion for modifying prior art to practice claimed invention, and evidence suggesting that invention could be successful, and reference thus rendered invention obvious to those of ordinary skill in art at time invention was made.

2. Patentability/Validity — Obviousness — Evidence of (§115.0906)

Experimenters' use of heterologous gene coded for ribosomal RNA, which is not ordinarily translated, rather than gene coded for predetermined protein, in plasmid cloning vector for introduction into host bacteria in genetic engineering experiment, does not require finding that applicant's claimed method of producing predetermined protein in host bacteria through genetic engineering was not obvious in view of published paper describing experiment, particularly observation that hybrid messenger RNA produced by experiment was apparently translated into protein, since it would have been obvious and reasonable to conclude from such observation that if gene coded for ribosomal RNA produced "junk" or "nonsense" protein, then use of gene coded for predetermined protein would result in production of "useful" protein, as application claims.

3. Patentability/Validity — Obviousness — In general (§115.0901)

Rejection of patent application cannot be overturned on ground that examiner and Board of Patent Appeals and Interferences applied impermissible "obvious to try" standard, since assignment of error for application of such standard usually occurs when invention is made by varying all parameters or trying each of numerous choices until successful without indication in prior art as to which parameters were critical or which choices were likely to be successful, or when invention is made by exploring promising new technology or general approach with only general guidance from prior art as to particular form of claimed invention or how to achieve it, and since neither situation is present in instant case.

4. Patentability/Validity — Obviousness — In general (§115.0901)

Finding of obviousness under 35 USC 103 requires only that prior art reveal reasonable expectation of success in producing claimed invention, rather than absolute prediction of such success.

Appeal from decision of Patent and Trademark Office, Board of Patent Appeals and Interferences.

Patent application, serial no. 180,424, filed by Patrick H. O'Farrell, Barry O. Polisky, and David H. Gelfand. From decision of Board of Patent Appeals and Interferences affirming final rejection of application on grounds of obviousness, applicants appeal. Affirmed.

J. Bruce McCubbrey of Fitch, Even, Tabin & Flannery (Virginia H. Meyer, with them on brief), San Francisco, Calif., for appellant.

Harris A. Pitlick, associate solicitor, Patent and Trademark Office (Joseph F. Nakamura, solicitor and Fred E. McKelvey, deputy solicitor, with him on brief), for appellee.

Before Markey, chief judge, and Rich and Nies, circuit judges.

Rich, J.

This appeal is from the decision of the United States Patent and Trademark Office Board of Patent Appeals and Interferences (board) affirming the patent examiner's final rejection of patent application Serial No. 180,424, entitled "Method and Hybrid Vector for Regulating Translation of heterologous DNA in Bacteria." The application was rejected under 35 USC 103 on the ground that the claimed invention would have been obvious at the time the invention was made in view of a published paper by two of the three coinventors, and a publication by Bahl, Marians & Wu 1 *Gene* 81 (1976) (Bahl). We affirm.

The claimed invention is from the developing new field of genetic engineering. A broad claim on appeal reads:

Claim 1. A method for producing a predetermined protein in a stable form in a transformed host species of bacteria comprising, providing a cloning vector which includes at least a substantial portion of a gene which is indigenous to the host species of bacteria and is functionally transcribed and translated in that species, said substantial portion of said indigenous gene further including the regulatory DNA sequences for RNA synthesis and protein synthesis but lacking the normal gene termination signal, and linking a natural or synthetic heterologous gene encoding said predetermined protein to said indigenous gene portion at its distal end, said heterologous gene being in proper orientation and having codons arranged in the same reading frame as the codons of said indigenous gene so that readthrough can occur from said indigenous gene portion into said heterologous gene in the same reading frame, said heterologous gene portion further containing sufficient DNA sequences to result in expression of a fused protein having sufficient size so as to confer stability on said predetermined protein when said vector is used to transform said host species of bacteria.

Illustrative embodiments are defined in more specific claims. For example:

Claim 2. A method for producing a predetermined protein in a stable form in a transformed host species of bacteria, comprising, providing an *E. coli* plasmid having an operator, a promoter, a site for the initiation of translation, and at least a substantial portion of the beta-galactosidase gene of the *E. coli* lactose operon, said substantial portion of said beta-galactosidase gene being under the control of said operator, promoter and site for initiation of translation, said substantial portion of said beta-galactosidase gene lacking the normal gene termination signal, and linking a heterologous gene encoding said predetermined protein to said beta-galactosidase gene portion at its distal end, said heterologous gene being in proper orientation and having codons arranged in the same reading frame as the codons of the said beta-galactosidase gene portion so that readthrough can occur from said beta-galactosidase gene portion into said heterologous gene in the same reading frame, said heterologous gene portion further containing sufficient DNA sequences to result in expression of a fused protein having sufficient size so as to confer stability on said predetermined protein when said vector is used to transform said host species of bacteria.

Claim 3. The method of Claim 2 wherein said *E. coli* plasmid comprises the plasmid designated pBGP120.

Although the terms in these claims would be familiar to those of ordinary skill in genetic engineering, they employ a bewildering vocabulary new to those who are not versed in molecular biology. An understanding of the science and technology on which these claims are based is essential before one can analyze and explain whether the claimed invention would have been obvious in light of the prior art.

I. *Background* [1]

Proteins are biological molecules of enormous importance. Proteins include enzymes that catalyze biochemical reactions, major structural materials of the animal body, and many hormones. Numerous patents and applications for patents in the field of biotechnology involve specific proteins or methods for making and using proteins. Many valuable proteins occur in nature only in minute quantities, or are difficult to purify from natural sources. Therefore, a goal of many biotechnology projects, including appellants' claimed invention, is to devise methods to synthesize useful quantities of specific proteins by controlling the mechanism by which living cells make proteins.

The basic organization of all proteins is the same. Proteins are large polymeric molecules consisting of chains of smaller building blocks, called *amino acids*, that are linked together covalently.[2] The chemical bonds linking amino acids together are called *peptide* bonds, so proteins are also called *poly-*

[1] Basic background information about molecular biology and genetic engineering, can be found in Alberts, Bray, Lewis, Raff, Roberts & Watson, *The Molecular Biology of the Cell*, 1-253, 385-481 (1983) [hereinafter *The Cell*]; Watson, Hopkins, Roberts, Steitz & Weiner, *The Molecular Biology of the Gene*, Vol. 1 (4th ed., 1987) 3-502 [hereinafter *The Gene*]. These standard textbooks were used to supplement the information in the glossary supplied by appellants. The description here is necessarily simplified and omits important facts and concepts that are not necessary for the analysis of this case.

[2] There are twenty amino acids: alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan, glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine, aspartic acid, glutamic acid, lysine, arginine, and histidine.

peptides.[3] It is the exact sequence in which the amino acids are strung together in a polypeptide chain that determines the identity of a protein and its chemical characteristics.[4] Although there are only 20 amino acids, they are strung together in different orders to produce the hundreds of thousands of proteins found in nature.

To make a protein molecule, a cell needs information about the sequence in which the amino acids must be assembled. The cell uses a long polymeric molecule, DNA (deoxyribonucleic acid), to store this information. The subunits of the DNA chain are called *nucleotides*. A nucleotide consists of a nitrogen-containing ring compound (called a *base*) linked to a 5-carbon sugar that has a phosphate group attached.[5] DNA is composed of only four nucleotides. They differ from each other in the base region of the molecule. The four bases of these subunits are adenine, guanine, cytosine, and thymine (abbreviated respectively as A, G, C and T). The sequence of these bases along the DNA molecule specifies which amino acids will be inserted in sequence into the polypeptide chain of a protein.

DNA molecules do not participate directly in the synthesis of proteins. DNA acts as a permanent "blueprint" of all of the genetic information in the cell, and exists mainly in extremely long strands (called *chromosomes*) containing information coding for the sequences of many proteins, most of which are not being synthesized at any particular moment. The region of DNA on the chromosome that codes for the sequence of a single polypeptide is called a *gene*.[6] In order to *express* a gene (the process whereby the information in a gene is used to synthesize new protein), a copy of the gene is first made as a molecule of RNA (ribonucleic acid).

RNA is a molecule that closely resembles DNA. It differs, however in that it contains a different sugar (ribose instead of deoxyribose) and the base thymine (T) of DNA is replaced in RNA by the structurally similar base, uracil (U). Making an RNA copy of DNA is called *transcription*. The transcribed RNA copy contains sequences of A, U, C, and G that carry the same information as the sequence of A, T, C, and G in the DNA. That RNA molecule, called *messenger RNA*, then moves to a location in the cell where proteins are synthesized.

The code whereby a sequence of nucleotides along an RNA molecule is translated into a sequence of amino acids in a protein (i.e., the "genetic code") is based on serially reading groups of three adjacent nucleotides. Each combination of three adjacent nucleotides, called a *codon*, specifies a particular amino acid. For example, the codon U-G-G in a messenger RNA molecule specifies that there will be a tryptophan molecule in the corresponding location in the corresponding polypeptide. The four bases A, G, C and U can be combined as triplets in 64 different ways, but there are only 20 amino acids to be coded. Thus, most amino acids are coded for by more than one codon. For example, both U-A-U and U-A-C code for tyrosine, and there are six different codons that code for leucine. There are also three codons that do not code for any amino acid (namely, U-A-A, U-G-A, and U-A-G). Like periods at the end of a sentence, these sequences signal the end of the polypeptide chain, and they are therefore called *stop codons*.

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[3] Proteins are often loosely called *peptides*, but technically proteins are only the larger peptides with chains of at least 50 amino acids, and more typically hundreds of amino acids. Some proteins consist of several polypeptide chains bound together covalently or noncovalently. The term "peptide" is broader than "protein" and also includes small chains of amino acids linked by peptide bonds, some as small as two amino acids. Certain small peptides have commercial or medical significance.

[4] Polypeptide chains fold up into complex 3-dimensional shapes. It is the shape that actually determines many chemical properties of the protein. However, the configuration of a protein molecule is determined by its amino acid sequence. *The Cell* at 111-12; *The Gene* at 50-54.

[5] The sugar in DNA is deoxyribose, while the sugar in RNA, *infra*, is ribose. The sugar and phosphate groups are linked covalently to those of adjacent nucleotides to form the backbone of the long unbranched DNA molecule. The bases project from the chain, and serve as the "alphabet" of the genetic code.

DNA molecules actually consist of two chains tightly entwined as a double helix. The chains are not identical but instead are complementary: each A on one chain is paired with a T on the other chain, and each C has a corresponding G. The chains are held together by noncovalent bonds between these complementary bases. This double helical structure plays an essential role in the replication of DNA and the transmission of genetic information. *See generally The Cell* at 98-106; *The Gene* at 65-79. However, the information of only one strand is used for directing protein synthesis, and it is not necessary to discuss the implication of the double-stranded structure of DNA here. RNA molecules, *infra*, are single stranded.

[6] Chromosomes also contain regions of DNA that are not part of genes, i.e., do not code for the sequence of amino acids in proteins. These include sections of DNA adjacent to genes that are involved in the control of transcription, *infra*, and regions of unknown function.

The cellular machinery involved in synthesizing proteins is quite complicated, and centers around large structures called *ribosomes* that bind to the messenger RNA. The ribosomes and associated molecules "read" the information in the messenger RNA molecule, literally shifting along the strand of RNA three nucleotides at a time, adding the amino acid specified by that codon to a growing polypeptide chain that is also attached to the ribosome. When a stop codon is reached, the polypeptide chain is complete and detaches from the ribosome.

The conversion of the information from a sequence of codons in an RNA molecule into the sequence of amino acids in a newly synthesized polypeptide is called *translation.* A messenger RNA molecule is typically reused to make many copies of the same protein. Synthesis of a protein is usually terminated by destroying the messenger RNA. (The information for making more of that protein remains stored in DNA in the chromosomes.)

The translation of messenger RNA begins at a specific sequence of nucleotides that bind the RNA to the ribosome and specify which is the first codon that is to be translated. Translation then proceeds by reading nucleotides, three at a time, until a stop codon is reached. If some error were to occur that shifts the frame in which the nucleotides are read by one or two nucleotides, all of the codons after this shift would be misread. For example, the sequence of codons [. . .*C-U-C-A-G-C-G-U-U-A-C-C-A*. . .] codes for the chain of amino acids [. . .leucine-serine-valine-threonine-. . .]. If the reading of these groups of three nucleotides is displaced by one nucleotide, such as [. . .*C-U-C-A-G-C-G-U-U-A-C-C-A*. . .], the resulting peptide chain would consist of [. . .serine-alanine-leucine-proline. . .]. This would be an entirely different peptide, and most probably an undesirable and useless one. Synthesis of a particular protein requires that the correct register or *reading frame* be maintained as the codons in the RNA are translated.

The function of messenger RNA is to carry genetic information (transcribed from DNA) to the protein synthetic machinery of a cell where its information is translated into the amino acid sequence of a protein. However, some kinds of RNA have other roles. For example, ribosomes contain several large strands of RNA that serve a structural function (*ribosomal RNA*). Chromosomes contain regions of DNA that code for the nucleotide sequences of structural RNAs and these sequences are transcribed to manufacture those RNAs. The DNA sequences coding for structural RNAs are still called genes even though the nucleotide sequence of the structural RNA is never translated into protein.

Man, other animals, plants, protozoa, and yeast are *eucaryotic* (or eukaryotic) organisms: their DNA is packaged in chromosomes in a special compartment of the cell, the nucleus. Bacteria (*procaryotic* or prokaryotic organisms) have a different organization. Their DNA, usually a circular loop, is not contained in any specialized compartment. Despite the incredible differences between them, all organisms, whether eucaryote or procaryote, whether man or mouse or lowly bacterium, use the same molecular rules to make proteins under the control of genes. In all organisms, codons in DNA are transcribed into codons in RNA which is translated on ribosomes into polypeptides according to the same genetic code. Thus, if a gene from a man is transferred into a bacterium, the bacterium can manufacture the human protein. Since most commercially valuable proteins come from man or other eucaryotes while bacteria are essentially little biochemical factories that can be grown in huge quantities, one strategy for manufacturing a desired protein (for example, insulin) is to transfer the gene coding for the protein from the eucaryotic cell where the gene normally occurs into a bacterium.

Bacteria containing genes from a foreign source (*heterologous* genes) integrated into their own genetic makeup are said to be *transformed.* When transformed bacteria grow and divide, the inserted heterologous genes, like all the other genes that are normally present in the bacterium (*indigenous* genes), are replicated and passed on to succeeding generations. One can produce large quantities of transformed bacteria that contain transplanted heterologous genes. The process of making large quantities of identical copies of a gene (or other fragment of DNA) by introducing it into procaryotic cells and then growing those cells is called *cloning* the gene. After growing sufficient quantities of the transformed bacteria, the biotechnologist must induce the transformed bacteria to *express* the cloned gene and make useful quantities of the protein. This is the purpose of the claimed invention.

In order to make a selected protein by expressing its cloned gene in bacteria, several technical hurdles must be overcome. First the gene coding for the specific protein must be isolated for cloning. This is a formidable task, but recombinant DNA technology has armed the genetic engineer with a variety of

techniques to accomplish it.[7] Next the isolated gene must be introduced into the host bacterium. This can be done by incorporating the gene into a cloning vector. A *cloning vector* is a piece of DNA that can be introduced into bacteria and will then replicate itself as the bacterial cells grow and divide. Bacteriophage (viruses that infect bacteria) can be used as cloning vectors, but plasmids were the type used by appellants. A *plasmid* is a small circular loop of DNA found in bacteria, separate from the chromosome, that replicates like a chromosome. It is like a tiny auxilliary chromosome containing only a few genes. Because of their small size, plasmids are convenient for the molecular biologist to isolate and work with. Recombinant DNA technology can be used to modify plasmids by splicing in cloned eucaryotic genes and other useful segments of DNA containing control sequences. Short pieces of DNA can even be designed to have desired nucleotide sequences, synthesized chemically, and spliced into the plasmid. One use of such chemically synthesized linkers is to insure that the inserted gene has the same reading frame as the rest of the plasmid; this is a teaching of the Bahl reference cited against appellants. A plasmid constructed by the molecular geneticist can be inserted into bacteria, where it replicates as the bacteria grow.

Even after a cloned heterologous gene has been successfully inserted into bacteria using a plasmid as a cloning vector, and replicates as the bacteria grow, there is no guarantee that the gene will be expressed, i.e., transcribed and translated into protein. A bacterium such as *E. coli* (the species of bacterium used by appellants) has genes for several thousand proteins. At any given moment many of those genes are not expressed at all. The genetic engineer needs a method to "turn on" the cloned gene and force it to be expressed. This is the problem appellants worked to solve.

II. *Prior art*

Appellants sought to control the expression of cloned heterologous genes inserted into bacteria. They reported the results of their early efforts in a publication, the three authors of which included two of the three coinventor-appellants (the Polisky reference [8]), that is undisputed prior art against them. Their strategy was to link the foreign gene to a highly regulated indigenous gene. Turning on expression of the indigenous gene by normal control mechanisms of the host would cause expression of the linked heterologous gene.

As a controllable indigenous gene, the researchers chose a gene in the bacterium *E. coli* that makes beta-galactosidase. *Beta-galactosidase* is an enzyme needed to digest the sugar, lactose (milk sugar). When *E. coli* grows in a medium that contains no lactose, it does not make beta-galactosidase. If lactose is added to the medium, the gene coding for beta galactosidase is expressed. The bacterial cell makes beta-galactosidase and is then able to use lactose as a food source. When lactose is no longer available, the cell again stops expressing the gene for beta galactosidase.

The molecular mechanisms through which the presence of lactose turns on expression of the beta-galactosidase gene has been studied in detail, and is one of the best understood examples of how gene expression is regulated on the molecular level. The beta-galactosidase gene is controlled by segments of DNA adjacent to the gene. These *regulatory DNA sequences* (the general term used in Claim 1) include the *operator* and *promoter* sequences (specified in Claim 2).[9] The researchers constructed a plasmid containing the beta-galactosidase gene with its operator and promoter. This gene (with its regulatory sequences) was removed from the chromosome of *E. coli* where it is normally found and was transplanted to a plasmid that could be conveniently manipulated.

Restriction endonucleases are useful tools in genetic engineering. These enzymes cut strands of DNA, but only at places where a specific sequence of nucleotides is present. For example, one restriction endonuclease, called *EcoRI*, cuts DNA only at sites where

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[7] See *The Cell* at 185–194; *The Gene* at 208–10.

[8] Polisky, Bishop & Gelfand, *A plasmid cloning vehicle allowing regulated expression of eukaryotic DNA in bacteria*, 73 Proc. Nat'l Acad. Sci. USA 3900 (1976).

[9] The *promoter* is a sequence of nucleotides where the enzyme that synthesizes RNA, *RNA polymerase*, attaches to the DNA to start the transcription of the beta-galactosidase gene. The *operator* is an overlapping DNA sequence that binds a small protein present in the cell, the lactose repressor protein. The lactose repressor protein binds to the operator and physically blocks the RNA polymerase from properly attaching to the promoter so that transcription cannot proceed. Lactose molecules interact with the lactose repressor protein and cause it to change its shape; after this change in shape it moves out of the way and no longer prevents the RNA polymerase from binding to the promoter. Messenger RNA coding for beta-galactosidase can then be transcribed. *See generally The Cell* at 438–39; *The Gene* at 474–80.

the nucleotide sequence is [. . .-G-A-A-T-T-C-. . .]. With restriction enzymes the genetic engineer can cut a strand of DNA at very specific sites into just a few pieces. With the help of "repair" enzymes, other pieces of DNA can be spliced onto the cut ends. The investigators found that the plasmid which they had constructed contained only two sequences that were cut by EcoRI. They were able to eliminate one of these sites that was unwanted. They were then left with a plasmid containing the beta-galactosidase gene with its regulatory sequences, and a single EcoRI site that was within the beta-galactosidase gene and close to its stop codon. They named this plasmid that they had constructed pBGP120.

The next step was to cut the plasmid open at its EcoRI site and insert a heterologous gene from another organism. The particular heterologous gene they chose to splice in was a segment of DNA from a frog that coded for ribosomal RNA. The frog gene was chosen as a test gene for reasons of convenience and availability. The new plasmid created by inserting the frog gene was similar to pBGP120, but its beta-galactosidase gene was incomplete. Some codons including the stop codon were missing from its end, which instead continued on with the sequence of the frog ribosomal RNA gene. The investigators named this new plasmid pBGP123. They inserted this plasmid back into *E. coli* and grew sufficient quantities for study. They then fed the *E. coli* with lactose. As they had intended, the lactose turned on transcription of the beta-galactosidase gene in the plasmid. RNA polymerase moved along the plasmid producing a strange new kind of RNA: Each long strand of RNA first contained codons for the messenger RNA for beta-galactosidase and then continued without interruption with the codons for the frog ribosomal RNA. Thus, there was *read-through* transcription in which the RNA polymerase first transcribed the indigenous (beta-galactosidase) gene and then "read through," i.e., continued into and through the adjacent heterologous (frog ribosomal RNA) gene. Although the RNA produced was a hybrid, it nevertheless contained a nucleotide sequence dictated by DNA from a frog. The researchers had achieved the first controlled transcription of an animal gene inside a bacterium.

The researchers had used a gene coding for a ribosomal RNA as their heterologous test gene. Ribosomal RNA is not normally translated into protein. Nevertheless, they were obviously interested in using their approach to make heterologous proteins in bacteria. They therefore examined the beta-galactosidase made by their transformed bacteria. Patrick O'Farrell, who was not a coauthor of the Polisky paper but was to become a coinventor in the patent application, joined as a collaborator. They found that beta-galactosidase from the transformed bacteria had a higher molecular weight than was normal. They concluded that the bacteria must have used their strange new hybrid RNA like any other messenger RNA and translated it into protein. When the machinery of protein synthesis reached the premature end of the sequence coding for beta-galactosidase it continued right on, three nucleotides at a time, adding whatever amino acid was coded for by those nucleotides, until a triplet was reached with the sequence of a stop codon. The resulting polypeptide chains had more amino acids than normal beta-galactosidase, and thus a higher molecular weight. The researchers published their preliminary results in the Polisky article. They wrote:

> [I]f the normal translational stop signals for [beta]-galactosidase are missing in pBGP120, in-phase translational readthrough into adjacent inserted sequences might occur, resulting in a significant increase in the size of the [beta]-galactosidase polypeptide subunit. In fact, we have recently observed that induced cultures of pBGP123 contain elevated levels of [beta]-galactosidase of higher subunit molecular weight than wild-type enzyme (P. O'Farrell, unpublished experiments). We believe this increase results from translation of *Xenopus* [frog] RNA sequences covalently linked to [messenger] RNA for [beta]-galactosidase, resulting in a fused polypeptide.

Polisky at 3904.

Since ribosomal RNA is never translated in normal cells, the polypeptide chain produced by translating that chain was not a naturally occurring, identified protein. The authors of the Polisky paper explicitly pointed out that if one were to insert a heterologous gene coding for a protein into their plasmid, it should produce a "fused protein" consisting of a polypeptide made of beta-galactosidase plus the protein coded for by the inserted gene, joined by a peptide bond into a single continuous polypeptide chain:

> It would be interesting to examine the expression of a normally translated eukaryotic sequence in pBGP120. If an inserted sequence contains a ribosome binding site that can be utilized in bacteria, production of high levels of a readthrough transcript might allow for extensive translation of a functional eukaryotic polypeptide. In the absence of an independent ribosome bind-

ing site, the eukaryotic sequence would be translated to yield a peptide covalently linked to [beta]-galactosidase. The extent of readthrough translation under *lac* control will depend on the number of translatable codons between the EcoRI site and the first in-phase nonsense [i.e., stop] codon in the inserted sequence.

Id.

III. *The Claimed Invention*

Referring back to Claims 1 through 3, it can be seen that virtually everything in the claims was present in the prior art Polisky article. The main difference is that in Polisky the heterologous gene was a gene for ribosomal RNA while the claimed invention substitutes a gene coding for a predetermined protein. Ribosomal RNA gene is not normally translated into protein, so expression of the heterologous gene was studied mainly in terms of transcription into RNA. Nevertheless, Polisky mentioned preliminary evidence that the transcript of the ribosomal RNA gene was translated into protein. Polisky further predicted that if a gene that codes for a protein were to be substituted for the ribosomal RNA gene, "a readthrough transcript might allow for extensive translation of a functional eukaryotic polypeptide." Thus, the prior art explicitly suggested the substitution that is the difference between the claimed invention and the prior art, and presented preliminary evidence suggesting that the method could be used to make proteins.

Appellants reduced their invention to practice some time in 1976 and reported their results in a paper that was published in 1978.[10] During 1977 they communicated their results to another group of researchers who used the readthrough translation approach to achieve the first synthesis of a human protein in bacteria.[11] Appellants filed an application to patent their invention on August 9, 1978, of which the application on appeal is a division.

IV. *The Obviousness Rejection*

The application was rejected under 35 USC 103. The position of the examiner and the Board is, simply, that so much of the appellant's method was revealed in the Polisky reference that making a protein by substituting its gene for the ribosomal RNA gene in Polisky (as suggested by Polisky) would have been obvious to one of ordinary skill in the art at the time that the invention was made.

The claims specify that the heterologous gene should be inserted into the plasmid in the same orientation and with the same reading frame as the preceding portion of the indigenous gene. In view of this limitation, the §103 rejection was based either on Polisky alone (supplemented by the fact that the importance of orientation and reading frame was well known in the prior art) or in combination with the Bahl reference which describes a general method for inserting a piece of chemically synthesized DNA into a plasmid. Bahl teaches that this technique could be used to shift the sequence of DNA inserted into a plasmid into the proper reading frame.

Appellants argue that at the time the Polisky article was published, there was significant unpredictability in the field of molecular biology so that the Polisky article would not have rendered the claimed method obvious to one of ordinary skill in the art. Even though there was speculation in the article that genes coding for proteins could be substituted for the ribosomal RNA gene and would be expressed as readthrough translation into the protein, this had never been done. Appellants say that it was not yet certain whether a heterologous protein could actually be produced in bacteria, and if it could, whether additional mechanisms or methods would be required. They contend

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[10] O'Farrell, Polisk & Gelfand, *Regulated expression by readthrough translation from a plasmid-encoded beta-galactosidase*, 134 J. Bacteriol. 645 (1978). The heterologous genes expressed in these studies were not predetermined, but were instead unidentified genes of unknown origin. The authors speculated that they were probably genes from *E. coli* that were contaminants in the source of beta-galactosidase genes. *Id.* at 648

[11] Itakura, Hirose, Crea, Riggs, Heynecker, Bolivar & Boyer, *Expression in Escherichia coli of a chemically synthesized gene for the hormone samatostatin*, 198 Science 1056 (1977). A pioneering accomplishment of the Itakura group is that the gene was not from a human source, but instead was entirely synthesized in the laboratory using chemical methods. It is not clear whether the appellants communicated only the results reported in the Polisky publication or whether they communicated the complete claimed invention.

that without such certainty the predictions in the Polisky paper, which hindsight now shows to have been correct, were merely invitations to those skilled in the art to try to make the claimed invention. They argue that the rejection amounts to the application of a standard of "obvious to try" to the field of molecular biology, a standard which this court and its predecessors have repeatedly rejected as improper grounds for a §103 rejection. *E.g., In re Fine*, 837 F.2d 1071, 1075, 5 USPQ2d 1596, 1599 (Fed. Cir. 1988); *In re Geiger*, 815 F.2d 686, 688, 2 USPQ2d 1276, 1278 (Fed. Cir. 1987); *In re Merck & Co., Inc.*, 800 F.2d 1091, 1097, 231 USPQ 375, 379 (Fed. Cir. 1986); *In re Antonie*, 559 F.2d 618, 620, 195 USPQ 6, 8 (CCPA 1977).

Obviousness under §103 is a question of law. *Panduit Corp. v. Dennison Mfg. Co.*, 810 F.2d 1561, 1568, 1 USPQ2d 1593, 1597 (Fed. Cir.), *cert. denied*, 107 S.Ct. 2187 (1987). An analysis of obviousness must be based on several factual inquiries: (1) the scope and content of the prior art; (2) the differences between the prior art and the claims at issue; (3) the level of ordinary skill in the art at the time the invention was made; and (4) objective evidence of nonobviousness, if any. *Graham v. John Deere Co.*, 383 U.S. 1, 17-18, 148 USPQ 459, 467 (1966). *See, e.g., Custom Accessories, Inc. v. Jeffrey-Allan Indus.*, 807 F.2d 955, 958, 1 USPQ2d 1196, 1197 (Fed. Cir. 1986). The scope and content of the prior art and the differences between the prior art and the claimed invention have been examined in sections II and III, *supra*. Appellants say that in 1976 those of ordinary skill in the arts of molecular biology and recombinant DNA technology were research scientists who had "extraordinary skill in relevant arts" and "were among the brightest biologists in the world." Objective evidence of nonobviousness was not argued.

[1] With the statutory factors as expounded by *Graham* in mind and considering all of the evidence, this court must determine the correctness of the board's legal determination that the claimed invention as a whole would have been obvious to a person having ordinary skill in the art at the time the invention was made. We agree with the board that appellants' claimed invention would have been obvious in light of the Polisky reference alone or in combination with Bahl within the meaning of §103. Polisky contained detailed enabling methodology for practicing the claimed invention, a suggestion to modify the prior art to practice the claimed invention, and evidence suggesting that it would be successful.

[2] Appellants argue that after the publication of Polisky, successful synthesis of protein was still uncertain. They belittle the predictive value of the observation that expression of the transcribed RNA in Polisky produced beta-galactosidase with a greater than normal molecular weight, arguing that since ribosomal RNA is not normally translated, the polypeptide chains that were added to the end of the beta-galactosidase were "junk" or "nonsense" proteins. This characterization ignores the clear implications of the reported observations. The Polisky study directly proved that a readthrough transcript messenger RNA had been produced. The preliminary observation showed that this messenger RNA was read and used for successful translation. It was well known in the art that ribosomal RNA was made of the same nucleotides as messenger RNA, that any sequence of nucleotides could be read in groups of three as codons, and that reading these codons should specify a polypeptide chain that would elongate until a stop codon was encountered. The preliminary observations thus showed that codons beyond the end of the beta-galactosidase gene were being translated into peptide chains. This would reasonably suggest to one skilled in the art that if the codons inserted beyond the end of the beta-galactosidase gene coded for a "predetermined protein," that protein would be produced. In other words, it would have been obvious and reasonable to conclude from the observation reported in Polisky that since nonsense RNA produced nonsense polypeptides, if meaningful RNA was inserted instead of ribosomal RNA, useful protein would be the result. The relative shortness of the added chains is also not a source of uncertainty, since one skilled in the art would have known that a random sequence of nucleotides would produce a stop codon before the chain got too long.[12]

Appellants complain that since predetermined proteins had not yet been produced in transformed bacteria, there was uncertainty as to whether this could be done, and that the rejection is thus founded on an impermissible "obvious to try" standard. It is true that this court and its predecessors have repeatedly emphasized that "obvious to try" is not the standard under §103. However, the meaning of this maxim is sometime lost. Any invention that would in fact have been obvious under §103 would also have been, in a sense, obvious to try. The question is: when is an

[12] The patent application indicates that chains as long as 60 amino acids were added, which is hardly a trivial length of polypeptide.

invention that was obvious to try nevertheless nonobvious?

[3] The admonition that "obvious to try" is not the standard under § 103 has been directed mainly at two kinds of error. In some cases, what would have been "obvious to try" would have been to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful. *E.g., In re Geiger*, 815 F.2d at 688, 2 USPQ2d at 1278; *Novo Industri A/S v. Travenol Laboratories, Inc.*, 677 F.2d 1202, 1208, 215 USPQ 412, 417 (7th Cir. 1982); *In re Yates*, 663 F.2d 1054, 1057, 211 USPQ 1149, 1151 (CCPA 1981); *In re Antonie*, 559 F.2d at 621, 195 USPQ at 8-9. In others, what was "obvious to try" was to explore a new technology or general approach that seemed to be a promising field of experimentation, where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it. *In re Dow Chemical Co.*, 837 F.2d, 469, 473, 5 USPQ2d 1529, 1532 (Fed. Cir. 1985); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1380, 231 USFQ 81, 90-91 (Fed. Cir. 1986), *cert. denied*, 107 S.Ct. 1606 (1987); *In re Tomlinson*, 363 F.2d 928, 931, 150 USPQ 623, 626 (CCPA 1966). Neither of these situations applies here.

[4] Obviousness does not require absolute predictability of success. Indeed, for many inventions that seem quite obvious, there is no absolute predictability of success until the invention is reduced to practice. There is always at least a possibility of unexpected results, that would then provide an objective basis for showing that the invention, although apparently obvious, was in law nonobvious. *In re Merck & Co.*, 800 F.2d at 1098, 231 USPQ at 380; *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1461, 221 USPQ 481, 488 (Fed. Cir. 1984); *In re Papesch*, 315 F.2d 381, 386-87, 137 USPQ 43, 47-48 (CCPA 1963). For obviousness under §103, all that is required is a reasonable expectation of success. *In re Longi*, 759 F.2d 887, 897, 225 USPQ 645, 651-52 (Fed. Cir. 1985); *In re Clinton*, 527 F.2d 1226, 1228, 188 USPQ 365, 367 (CCPA 1976). The information in the Polisky reference, when combined with the Bahl reference provided such a reasonable expectation of success.

Appellants published their pioneering studies of the expression of frog ribosomal RNA genes in bacteria more than a year before they applied for a patent. After providing virtually all of their method to the public without applying for a patent within a year, they foreclosed themselves from obtaining a patent on a method that would have been obvious from their publication to those of ordinary skill in the art, with or without the disclosures of other prior art. The decision of the board is

AFFIRMED.

District Court, W.D. Washington

Specialized Electronics Corp. v. Aviation Supplies & Academics Inc.

No. C86-712D

Decided March 23, 1988

PATENTS

1. Patentability/Validity — Obviousness — Secondary considerations (§115.0907)

Patent infringement defendant has failed to sustain its burden of proving, by clear and convincing evidence, that claims for hand-held aircraft navigational computers are invalid for obviousness under 35 USC 103, in view of objective evidence of secondary considerations demonstrating non-obviousness.

2. Infringement — Defenses — Prosecution history estoppel (§120.1105)

Doctrine of prosecution history estoppel applies to arguments narrowing construction of claims even if claims are not amended.

3. Patent construction — Claims — Broad or narrow (§125.1303)

Doctrine of claim differentiation precludes reading into independent claim limitation explicitly set forth in another claim, and such doctrine, although it is useful tool of claim construction, cannot be used to repudiate arguments made to Patent and Trademark Office in order to obtain allowance of asserted claims over prior art.

Particular patents — General and mechanical — Computers

3,979,057, Katz, Aronson, and Turek, self-contained hand-held electronic computer for aircraft navigation problems, claim 27 valid but not infringed.

3,979,058, Katz, Aronson, and Turek, self-contained electronic computer for math-

EXHIBIT 3

SUPPLEMENTAL AMENDMENT TO MAY 4, 2009 AMENDMENT
FILED IN RESPONSE TO NOVEMBER 3, 2008 OFFICE ACTION

Submitted: October 7, 2009

Serial No. 10/821,726

Filed: April 8, 2004

Applicants: Michael Wayne Graham et al.

▻

United States Court of Appeals,
Federal Circuit.
EISAI CO. LTD. and Eisai, Inc., Plaintiffs-Appellees,
v.
DR. REDDY'S LABORATORIES, LTD. and Dr. Reddy's Laboratories, Inc., Defendants-Appellants,
and
Teva Pharmaceuticals USA, Inc., Defendant-Appellant.
Nos. 2007-1397, 2007-1398.

July 21, 2008.
Rehearing and Rehearing En Banc Denied Sept. 16, 2008.

Background: Patentee of patent claiming lead compound used in pharmaceutical approved for the treatment of duodenal ulcers, heartburn, and associated disorders brought infringement action against competitors. The United States District Court for the Southern District of New York, Gerard E. Lynch, J., 472 F.Supp.2d 493, 2006 WL 2872615, granted in part and denied in part owner's motions for summary judgment, and found competitors infringed patent. Competitors appealed.

Holdings: The Court of Appeals, Rader, Circuit Judge, held that:
(1) prior art did not render patent obvious, and
(2) patentee did not commit inequitable conduct in prosecuting patent application for patent.

Affirmed.

West Headnotes

[1] Patents 291 ⚿16.13

291 Patents
291II Patentability
291II(A) Invention; Obviousness
291k16.13 k. Fact Questions. Most Cited Cases
Obviousness, for patent law purposes, is ultimately a legal question, based on underlying factual determinations. 35 U.S.C.A. § 103(a).

[2] Patents 291 ⚿16(2)

291 Patents
291II Patentability
291II(A) Invention; Obviousness
291k16 Invention and Obviousness in General
291k16(2) k. Prior Art in General. Most Cited Cases

Patents 291 ⚿16(3)

291 Patents
291II Patentability
291II(A) Invention; Obviousness
291k16 Invention and Obviousness in General
291k16(3) k. View of Person Skilled in Art. Most Cited Cases

Patents 291 ⚿36.1(1)

291 Patents
291II Patentability
291II(A) Invention; Obviousness
291k36 Weight and Sufficiency
291k36.1 Secondary Factors Affecting Invention or Obviousness
291k36.1(1) k. In General. Most Cited Cases
The factual determinations underpinning the legal conclusion of obviousness, for patent law purposes, include: (1) the scope and content of the prior art; (2) the level of ordinary skill in the art; (3) the differences between the claimed invention and the prior art; and (4) evidence of secondary factors, also known as objective indicia of non-obviousness. 35 U.S.C.A. § 103(a).

[3] Patents 291 ⚿324.5

291 Patents
291XII Infringement
291XII(C) Suits in Equity
291k324 Appeal
291k324.5 k. Scope and Extent of Review in General. Most Cited Cases
In reviewing a district court's summary judgment of non-obviousness in a patent infringement proceeding, the appellate court reviews the record for genuine issues of material fact without deference, bearing in mind the movant's burden to prove invalidity by clear and convincing evidence. 35 U.S.C.A. § 103(a).

[4] Patents 291 ⚿16.25

291 Patents
291II Patentability
291II(A) Invention; Obviousness
291k16.25 k. Chemical Compounds. Most Cited Cases
Where the patent at issue claims a chemical compound, the analysis of the third *Graham* factor for determining obviousness, the differences between the claimed invention and the prior art, often turns on the structural similarities and differences between the claimed compound and the prior art compounds; obviousness based on structural similarity thus can be proved by identification of some motivation that would have led one of ordinary skill in the art to select and then modify a known compound in a particular way to achieve the claimed compound. 35 U.S.C.A. § 103(a).

[5] Patents 291 ⚿16.25

291 Patents
291II Patentability
291II(A) Invention; Obviousness
291k16.25 k. Chemical Compounds. Most Cited Cases
The requisite motivation to prove the obviousness of a patent claiming a chemical compound based on structural similarity can come from any number of sources and need not necessarily be explicit in the art; rather it is sufficient to show that the claimed and prior art compounds possess a sufficiently close relationship to create an expectation, in light of the totality of the prior art, that the new compound will have similar properties to the old. 35 U.S.C.A. § 103(a).

[6] Patents 291 ⚿16.25

291 Patents
291II Patentability
291II(A) Invention; Obviousness
291k16.25 k. Chemical Compounds. Most Cited Cases
Prior art did not render obvious patent claiming lead compound used in pharmaceutical approved for the treatment of duodenal ulcers, heartburn, and associated disorders, where compounds claimed by prior art differed structurally from compound claimed by patent, and the record contained no reasons a skilled artisan would have considered the differences between the compounds identifiable and predictable. 35 U.S.C.A. § 103(a).

[7] Patents 291 ⚿324.54

291 Patents
291XII Infringement
291XII(C) Suits in Equity
291k324 Appeal
291k324.54 k. Presumptions and Discretion of Lower Court. Most Cited Cases

Patents 291 ⚿324.55(2)

291 Patents
291XII Infringement
291XII(C) Suits in Equity
291k324 Appeal
291k324.55 Questions of Fact, Verdicts, and Findings
291k324.55(2) k. Clearly Erroneous Findings. Most Cited Cases
Where a judgment regarding inequitable conduct in prosecuting a patent application follows a bench trial, the appellate court reviews the district court's findings of materiality and intent for clear error and its ultimate conclusion for an abuse of discretion.

[8] Patents 291 ⚿97

291 Patents

291IV Applications and Proceedings Thereon
291k97 k. Patent Office and Proceedings Therein in General. Most Cited Cases
Inequitable conduct in prosecuting a patent application before the Patent and Trademark Office (PTO) may take the form of an affirmative misrepresentation of material fact, a failure to disclose material information, or the submission of false material information, but in every case this false or misleading material communication or failure to communicate must be coupled with an intent to deceive; "materiality," defined as "what a reasonable examiner would have considered important in deciding whether to allow a patent application," and intent are both questions of fact, and require proof by clear and convincing evidence.

[9] Patents 291 ⚿97

291 Patents
291IV Applications and Proceedings Thereon
291k97 k. Patent Office and Proceedings Therein in General. Most Cited Cases
To satisfy the "intent" prong for unenforceability of a patent due to inequitable conduct during the prosecution of a patent application, the involved conduct, viewed in light of all the evidence, including evidence indicative of good faith, must indicate sufficient culpability to require a finding of intent to deceive; gross negligence is not sufficient.

[10] Patents 291 ⚿97

291 Patents
291IV Applications and Proceedings Thereon
291k97 k. Patent Office and Proceedings Therein in General. Most Cited Cases
Patentee did not commit inequitable conduct in prosecuting patent application for patent claiming lead compound used in pharmaceutical approved for the treatment of duodenal ulcers, heartburn, and associated disorders by failing to disclose its own co-pending application, withholding rejections from its co-pending application that also would have applied to patent, failing to disclose prior art, submitting a misleading declaration, and concealing similar compound, where record lacked sufficient evidence of intent to deceive.

Patents 291 ⚿328(2)

291 Patents
291XIII Decisions on the Validity, Construction, and Infringement of Particular Patents
291k328 Patents Enumerated
291k328(2) k. Original Utility. Most Cited Cases
4,255,431. Cited as Prior Art.

Patents 291 ⚿328(2)

291 Patents
291XIII Decisions on the Validity, Construction, and Infringement of Particular Patents
291k328 Patents Enumerated
291k328(2) k. Original Utility. Most Cited Cases
5,045,552. Infringed.
***1355** Joseph M. O'Malley, Jr., Paul, Hastings, Janofsky & Walker, LLP, of New York, New York, argued for plaintiffs-appellees. With him on the brief were Bruce M. Wexler, David M. Conca, Gary G. Ji, and Quinn E. Clancy.

Maurice N. Ross, Budd Larner, P.C., of Short Hills, New Jersey, argued for defendants-appellants Dr. Reddy's Laboratories, Ltd., and Dr. Reddy's Laboratories, Inc. With him on the brief were Andrew J. Miller, Louis H. Weinstein, Ellen T. Lowenthal, and Dmitry V. Sheluho.

Henry C. Dinger, Goodwin Procter LLP, of Boston, Massachusetts, argued for defendant-appellant Teva Pharmaceuticals USA, Inc. With him on the brief were Elaine H. Blais, and David M. Hashmall, Frederick H. Rein, and Emily L. Rapalino, of New York, New York.

Before RADER, LINN, and PROST, Circuit Judges.

RADER, Circuit Judge.

On summary judgment, the United States District Court for the Southern District of New York found in favor of plaintiffs Eisai Co., Ltd. and Eisai, Inc. (collectively Eisai) with respect to the validity and en-

forceability of U.S. Patent No. 5,045,552 ('552 patent). *Eisai Co. v. Teva Pharms. USA, Inc.*, 472 F.Supp.2d 493 (S.D.N.Y.2006) (*SJ Validity Order*); *Eisai Co. v. Dr. Reddy's Labs., Ltd.*, No. 03 Civ. 9053 (S.D.N.Y. Oct. 5, 2006) (*SJ Enforceability Order*). After a bench trial, the district court found that Dr. Reddy's Laboratories, Ltd. and Dr. Reddy's Laboratories, Inc. (collectively Dr. Reddy's) and Teva Pharmaceuticals USA, Inc. (Teva) had failed to prove the remaining allegations of inequitable conduct, and that Eisai had established that Dr. Reddy's and Teva infringed Eisai's '552 patent. *Eisai Co. v. Dr. Reddy's Labs., Ltd.*, No. 03 Civ. 9053, 2007 WL 1406565 (S.D.N.Y. May 11, 2007) (*Trial Order*). Because the district court correctly determined that the '552 patent is non-obvious over the proffered prior art and that Eisai's alleged acts during prosecution did not rise to the level of inequitable conduct, this court affirms.

***1356 I**

The '552 patent claims rabeprazole and its salts. Rabeprazole is part of a class of drugs known as proton pump inhibitors, which suppress gastric acid production by inhibiting action of the enzyme H^+K^+ATPase. The distinctions between rabeprazole and its salts are not relevant for this appeal. Therefore this court refers to rabeprazole and its salts collectively as "rabeprazole." Rabeprazole's sodium salt is the active ingredient in Aciphex, a pharmaceutical approved in 1991 by the FDA for the treatment of duodenal ulcers, heartburn, and associated disorders. Aciphex has been a commercial success, garnering over $1 billion in worldwide yearly sales.

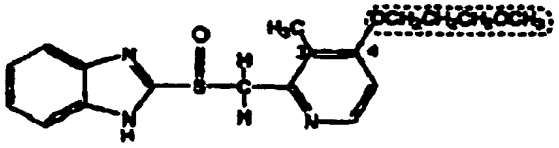
Dr. Reddy's and Teva each filed Abbreviated New Drug Applications (ANDAs) under the Hatch-Waxman Act, 21 U.S.C. § 355 and 35 U.S.C. § 271(e), seeking to manufacture a generic version of Aciphex before the expiration of the '552 patent. Because filing an ANDA is an artificial, but legally cognizable, act of patent infringement, *see Glaxo Group Ltd. v. Apotex, Inc.*, 376 F.3d 1339, 1344 (2004), Eisai filed suit against Dr. Reddy's and Teva. Eisai also sued Mylan Laboratories Inc. and Mylan Pharmaceuticals Inc. (collectively Mylan), another ANDA filer, but that proceeding was stayed pending the outcome of these actions. Mylan agreed to be bound by the final judgments and any appeals in these cases. *Eisai Co., Ltd. v. Mylan Labs., Inc.*, No. 04 Civ. 656 (S.D.N.Y. Nov. 3, 2004). Both Dr. Reddy's and Teva conceded infringement of claims 1-6 of the '552 patent, but asserted that the '552 patent is unenforceable for inequitable conduct. *Trial Order* at 6-7. Dr. Reddy's stipulated to the validity of all six of the '552 patent's claims, *id.* at 6, but Teva argued before the district court and maintains on appeal that the '552 patent is invalid for obviousness. Both Dr. Reddy's and Teva appeal the trial court's judgments of enforceability. Neither Dr. Reddy's nor Teva appeals the trial court's judgment of infringement. This court has jurisdiction under 28 U.S.C. § 1295(a)(1).

II

[1][2][3] This court reviews a grant of summary judgment without deference. *Dayco Prods., Inc. v. Total Containment, Inc.*, 329 F.3d 1358, 1362 (Fed.Cir.2003). Obviousness under 35 U.S.C. § 103(a) is ultimately a legal question, based on underlying factual determinations. *See Richardson-Vicks Inc. v. Upjohn Co.*, 122 F.3d 1476, 1479 (Fed.Cir.1997). The factual determinations underpinning the legal conclusion of obviousness include 1) the scope and content of the prior art, 2) the level of ordinary skill in the art, 3) the differences between the claimed invention and the prior art, and 4) evidence of secondary factors, also known as objective indicia of non-obviousness. *Graham v. John Deere Co.*, 383 U.S. 1, 17-18, 86 S.Ct. 684, 15 L.Ed.2d 545 (1966). Thus, in reviewing a district court's summary judgment of non-obviousness, this court reviews the record for genuine issues of material fact without deference, bearing in mind the movant's burden to prove invalidity by clear and convincing evidence. *See Monarch Knitting Mach. Corp. v. Sulzer Morat GmbH*, 139 F.3d 877, 881 (Fed.Cir.1998).

[4][5] Where, as here, the patent at issue claims a chemical compound, the analysis of the third *Graham* factor (the differences between the claimed invention and the prior art) often turns on the structural similarities and differences between the claimed compound and the prior art ***1357** compounds. *See Eli Lilly & Co. v. Zenith Goldline Pharms., Inc.*, 471 F.3d 1369, 1377 (Fed.Cir.2006) (noting that, for a chemical compound, a prima facie case of obviousness requires "structural similarity between claimed and prior art

subject matter ... where the prior art gives reason or motivation to make the claimed compositions" (quoting *In re Dillon*, 919 F.2d 688, 692 (Fed.Cir.1990) (en banc))). Obviousness based on structural similarity thus can be proved by identification of some motivation that would have led one of ordinary skill in the art to select and then modify a known compound (i.e. a lead compound) in a particular way to achieve the claimed compound. *See Takeda Chem. Indus. v. Alphapharm Pty., Ltd.*, 492 F.3d 1350, 1356 (Fed.Cir.2007). In keeping with the flexible nature of the obviousness inquiry, *KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. 398, 127 S.Ct. 1727, 1739, 167 L.Ed.2d 705 (2007), the requisite motivation can come from any number of sources and need not necessarily be explicit in the art. *See Aventis Pharma Deutschland GmbH v. Lupin, Ltd.*, 499 F.3d 1293, 1301 (Fed.Cir.2007). Rather "it is sufficient to show that the claimed and prior art compounds possess a 'sufficiently close relationship ... to create an expectation,' in light of the totality of the prior art, that



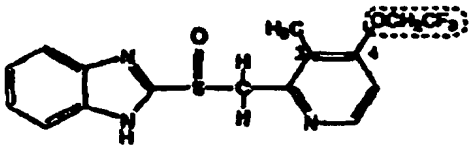
Rabeprazole

Appellant Teva's Br. at 28. Otherwise, the two compounds are identical. *See SJ Validity Order* at 7. Both rabeprazole and lansoprazole are "asymmetrically substituted" with respect to the 4-position on the pyridine ring because the substituent at the 3-position (a methyl group in both compounds) is not the same as the substituent at the 5-position (a hydrogen in both compounds).

The '431 patent discloses a broad class of gastric acid inhibiting compounds, including omeprazole, the first commercial proton pump inhibitor, sold as Prilosec. Although sharing the same basic structure, omeprazole is structurally farther afield from rabeprazole than is lansoprazole. For instance, omeprazole's pyridine ring is symmetrically substituted and has a methoxy (OCH3) group at the 4-position.

the new compound will have 'similar properties' to the old." *Id.* (quoting *Dillon*, 919 F.2d at 692).

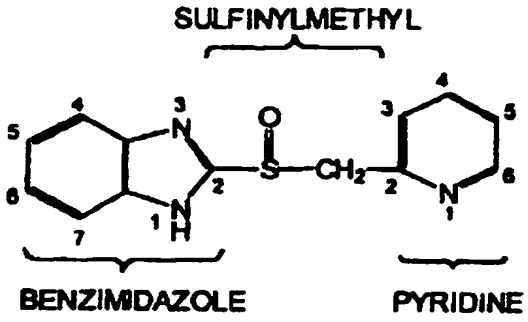
[6] Teva asserts that a combination of three prior art references renders the '552 patent obvious: 1) European Patent No. 174,726 (owned by Takeda), claiming lansoprazole (EP '726); 2) United States Patent No. 4,255,431 (to Junggren), claiming omeprazole ('431 patent); and 3) an article by Brändström, et al., entitled "Structure Activity Relationships of Substituted Benzimidazoles" (Brändström). EP '726 teaches, inter alia, the ulcer treatment le. Lansoprazole differs structurally from rabeprazole at the 4-position on the pyridine ring, as indicated in the diagram below. Lansoprazole has a trifluoroethoxy (OCH_2CF_3) substituent, whereas rabeprazole has a methoxypropoxy ($OCH_2CH_2CH_2\ OCH_3$) substituent.



Lansoprazole

Finally, Brändström describes a class of anti-ulcerative compounds having a benzimidazole-sulfinylmethyl-pyridine core (the Brändström core structure):

***1358**



Brändström Core Structure

Rabeprazole, lansoprazole, and omeprazole are all Brändström core structure compounds. Taking the evidence in the light most favorable to Teva, this court assumes that as per EP '726, lansoprazole is twenty times superior to omeprazole for anti-ulcer action, as measured by an indomethacin-induced gastric lesion assay in rats. This court also assumes that lansoprazole has certain traits, including lipophilicity (the ability of a compound to cross lipid membranes) and low molecular weight, that would have made it desirable to a skilled artisan.

Under these assumptions, one of skill in this art may have considered it a candidate for a lead compound in the search for anti-ulcer compounds. To the contrary, the district court emphasized the differences between anti-ulcer action and gastric acid inhibition. The trial court specifically noted that Teva's expert testified with respect to the EP '726 data that "[t]he level of acid secretion ... from these [anti-ulcer] data ... cannot be determined." *SJ Validity Order* at 13. In this context, this court consults the counsel of *KSR* that "any need or problem known in the field of endeavor at the time of invention and addressed by the patent can provide a reason for combining the elements in the manner claimed." 127 S.Ct. at 1742. Thus lansoprazole's candidacy as a starting point to develop new anti-ulcer compounds versus new gastric acid inhibitors does not resolve the lead compound analysis, at least not in the absence of any contrary indications. *Cf. Takeda*, 492 F.3d at 1359 (negative side effects could dissuade one of skill from using a particular compound as a starting point).

Nonetheless, as the district court noted, the EP '726 reference teaches at best that the fluorinated substituent of lansoprazole provides "a *special path* to achieving lipophilicity." *SJ Validity Order* at 10 (emphasis in original). And Teva's expert identified a separate reference teaching that fluorine-substituted groups increase lipophilicity. *Id.* The record, however, shows no discernible reason for a skilled artisan to begin with lansoprazole only to drop the very feature, the fluorinated substituent, that gave this advantageous property. Indeed, Teva's pharmacology expert, Dr. John Forte, declined to opine on lansoprazole's relevance to an examiner assessing the patentability of rabeprazole. J.A. at 14894. And Dr. Reddy's pharmacology expert, Dr. Simmy Bank, testified in deposition that "I thought [lansoprazole] had nothing to do with this trial." J.A. at 14756.

This court notes that the district court did not rigidly limit Teva's obviousness arguments by forcing Teva to select a single lead compound. Rather Teva alone ***1359** selected lansoprazole as the anchor for its obviousness theory, not the district court. In *KSR*, the Supreme Court noted that an invention may have been obvious "[w]hen there [was] ... a design need or market pressure to solve a problem and there [were] ... a finite number of identified, predictable solutions." 127 S.Ct. at 1742 (tense changes supplied to clarify, as the Court stated and as per 35 U.S.C. § 103, that the obviousness inquiry must rely on evidence available "at the time" of the invention, *see Takeda*, 492 F.3d at 1356 n. 2). The Supreme Court's analysis in *KSR* thus relies on several assumptions about the prior art landscape. First, *KSR* assumes a starting reference point or points in the art, prior to the time of

invention, from which a skilled artisan might identify a problem and pursue potential solutions. Second, *KSR* presupposes that the record up to the time of invention would give some reasons, available within the knowledge of one of skill in the art, to make particular modifications to achieve the claimed compound. *See Takeda*, 492 F.3d at 1357 ("Thus, in cases involving new chemical compounds, it remains necessary to identify some reason that would have led a chemist to modify a known compound in a particular manner to establish prima facie obviousness of a new claimed compound."). Third, the Supreme Court's analysis in *KSR* presumes that the record before the time of invention would supply some reasons for narrowing the prior art universe to a "finite number of identified, predictable solutions," 127 S.Ct. at 1742. In *Ortho-McNeil Pharmaceutical, Inc. v. Mylan Laboratories, Inc.*, 520 F.3d 1358, 1364 (Fed.Cir.2008), this court further explained that this "easily traversed, small and finite number of alternatives ... might support an inference of obviousness." To the extent an art is unpredictable, as the chemical arts often are, *KSR*'s focus on these "identified, predictable solutions" may present a difficult hurdle because potential solutions are less likely to be genuinely predictable.

In other words, post- *KSR*, a prima facie case of obviousness for a chemical compound still, in general, begins with the reasoned identification of a lead compound. Teva cannot create a genuine issue of material fact on obviousness through the unsupported assertion that compounds other than lansoprazole might have served as lead compounds. Further, the record contains no reasons a skilled artisan would have considered modification of lansoprazole by removing the lipophilicity-conferring fluorinated substituent as an identifiable, predictable solution. In sum, the district court properly concluded that the record did not support a case of obviousness of the '552 patent as a matter of law.

III

[7] As with other summary judgment issues, this court reviews a district court's summary judgment on inequitable conduct without deference. *Innogenetics, N.V. v. Abbott Labs.*, 512 F.3d 1363, 1378 (Fed.Cir.2008). In contrast, where a judgment regarding inequitable conduct follows a bench trial, this court reviews the district court's findings of materiality and intent for clear error and its ultimate conclusion for an abuse of discretion. *ACCO Brands, Inc. v. ABA Locks Mfrs. Co.*, 501 F.3d 1307, 1314 (Fed.Cir.2007).

[8][9] Inequitable conduct in prosecuting a patent application before the United States Patent & Trademark Office may take the form of an affirmative misrepresentation of material fact, a failure to disclose material information, or the submission***1360** of false material information, but in every case this false or misleading material communication or failure to communicate must be coupled with an intent to deceive. *Innogenetics*, 512 F.3d at 1378 (citations omitted). Materiality, defined as "what a reasonable examiner would have considered important in deciding whether to allow a patent application," and intent are both questions of fact, and require proof by clear and convincing evidence. *Id.* To satisfy the "intent" prong for unenforceability, "the involved conduct, viewed in light of all the evidence, including evidence indicative of good faith, must indicate sufficient culpability to require a finding of intent to deceive." *Kingsdown Med. Consultants, Ltd. v. Hollister Inc.*, 863 F.2d 867, 876 (Fed.Cir.1988) (en banc) (citing *Norton v. Curtiss*, 57 C.C.P.A. 1384, 433 F.2d 779 (1970)). Gross negligence is not sufficient. *Id.* This is a high bar.

[10] On appeal, Teva and Dr. Reddy's allege that Eisai misled the Patent Office in five ways: 1) failing to disclose Eisai's own co-pending '013 application, which claimed the "ethyl homolog" of rabeprazole (compound SHKA 661); 2) withholding rejections from the '013 application's prosecution that also would have been applicable to the '552 patent's prosecution; 3) failing to disclose the prior art "Byk Gulden patent" (WO 8602646); 4) submitting a misleading declaration (the Fujisaki Declaration) to the examiner of the '552 patent; and 5) concealing lansoprazole from the examiner. The district court rejected the fifth assertion on summary judgment, *SJ Enforceability Order* at 58, and the other four after a bench trial, *Trial Order*.

Teva and Dr. Reddy's first and second allegations rely on Eisai's failure to disclose the fact of, and rejections contained in, Eisai's patent application claiming the "ethyl homolog" of rabeprazole. Known to Eisai's

scientists as compound SHKA 661, the ethyl homolog differs from rabeprazole as its name suggests. SHKA 661 has one fewer methylene unit at the 4-position of the pyridine ring, giving SHKA 661 an ethoxy group rather than a propoxy group at this position. The district court correctly pointed out that calling SHKA 661 the "ethyl homolog" of rabeprazole in this case could carry a misleading implication with respect to inequitable conduct. The record supplies no evidence to suggest that Eisai's scientists ever referred to SHKA 661 by this name, or thought of SHKA 661 and rabeprazole "primarily in relation to each other." *Trial Order* at 17 n. 7. Rather, the district court found credible the testimony that Eisai scientists considered SHKA 661 separately patentable, even though Eisai ultimately did not pursue that course. *Id.* at 22-23; 42-43. Furthermore, even if a provisional obviousness-type double-patenting rejection might have issued in the prosecution of the '552 patent due to the co-pending SHKA 661 application, the district court found the materiality of this potential situation low, because applicants routinely overcome this type of rejection, *id.* at 44, by amending claims or filing a terminal disclaimer. Nonetheless, the district court did not hold that the fact of the copendency of these two applications to be totally immaterial, accurately noting that applicants should be encouraged to disclose closely related applications. *Id.* at 47.

While disclosure of the co-pending SHKA 661 application to the Patent Office during the prosecution of the '552 patent would have been prudent, Eisai's failure to do so is by no means fatal, for two reasons. First, the district court had ample evidence from which to conclude that the materiality of the SHKA 611 application ***1361** was low, as outlined above. Second, the record is devoid of any real suggestion of intent to deceive the Patent Office, much less the clear and convincing evidence required to support a finding of inequitable conduct.

As for the rejections of the '013 application that would have been relevant to the prosecution of the '552 patent, the district court did not reach materiality because it discerned insufficient proof of intent to deceive. The district court found the documentary evidence (faxed exchange between Eisai employees Mr. Shuhei Miyazawa, one of the inventors of the '552 patent, and Mr. Mitsuo Taniguchi, Eisai's patent agent, regarding Mr. Miyazawa's presentation to a pharmaceutical trade industry group) to supply no compelling evidence of intent, based on testimony from both parties to the fax. Witness credibility determinations lie squarely within the district court's discretion. *See Medichem, S.A. v. Rolabo, S.L.*, 437 F.3d 1157, 1171 (Fed.Cir.2006). The district court was ultimately undisturbed by the Taniguchi/Miyazawa communication based on its evaluation of the witness testimony presented, and this court sees no abuse of discretion. These facts certainly do not rise to the level of "culpability" this court required in *Kingsdown*, 863 F.2d at 876, to establish intent to deceive, or even gross negligence.

Finally, the district court found that Teva's theory that Eisai deliberately hid the ball from the Patent Office by separately filing the '552 and '013 prosecutions to be "implausibly risky," given that such similar applications would usually be assigned to the same examiner in the same art unit. *Trial Order* at 53. The district court thus had ample bases from which to conclude that Eisai's failure to disclose its co-pending '013 application along with the rejections issued in its prosecution, while not completely forthcoming, did not rise to the level of inequitable conduct.

With respect to the Byk Gulden patent, Teva and Dr. Reddy's argue that Eisai's failure to disclose this reference to the Patent Office during prosecution of the '552 patent was material because a reasonable examiner would have used it to issue a new and stronger prima facie obviousness rejection on the basis of Byk Gulden's disclosure of asymmetrically-substituted compounds having a methoxyethoxy at the 4-position of the pyridine ring. But the district court found Byk Gulden's teachings cumulative with references already disclosed to the Patent Office (Junggren or Junggren combined with Beecham). As per 37 C.F.R. § 1.56, cumulative evidence is definitionally not material evidence. *See Monsanto Co. v. Bayer Bioscience N.V.*, 514 F.3d 1229, 1237 (Fed.Cir.2008). Here, the Junggren reference specifically disclosed asymmetrically substituted compounds, including a compound having a 4-position methoxyethoxy substituent. Thus the Byk Gulden reference offered nothing new to the record already before the Patent Office. And even Teva's expert conceded Byk Gulden would not have provided the examiner with anything new. *Id.* at 57.

Thus the district court was well within its discretion in concluding that the Byk Gulden patent was not material to the prosecution of the '552 patent. Even if Byk Gulden had been material, the lack of clear and convincing evidence of intent to deceive would nonetheless have imposed an insurmountable bar to finding inequitable conduct, for the reasons given by the district court.

As for the Fujisaki Declaration, Eisai submitted it during prosecution to overcome an obviousness rejection. Because this reference shows rabeprazole's pharmacological properties, the trial court found it highly material. *Id.* at 59. Teva ***1362** and Dr. Reddy's argue that the data presented in the Fujisaki Declaration were misleading. They contend that the comparison with two non-prior art compounds without a comparison of the ethyl homolog of rabeprazole, SHKA 661, sent the examiner on a dead-end side trip. The district court properly characterized this argument as "contorted." *Id.* The Fujisaki Declaration indisputably showed a comparison between rabeprazole and the prior art compound called out by the examiner, demonstrating rabeprazole's superiority. Further, as discussed above, the materiality of SHKA 661 and the patent application claiming it was low. The data from the Fujisaki Declaration were relevant to prosecution, but Eisai had no obligation to include additional, unnecessary data such as a comparison to SHKA 661. Thus the district court did not abuse its discretion in concluding that Eisai did not commit inequitable conduct in failing to include additional data in the Fujisaki Declaration to the examiner. Even here, where the submission to the Patent Office itself was highly material to prosecution, the lack of deceptive intent rendered stillborn yet another allegation of inequitable conduct.

Finally, Teva and Dr. Reddy's assert that that Eisai deceptively declined to inform the examiner of a patent application for lansoprazole, a prior art proton pump inhibitor (and the active ingredient in Prevacid). The district court disposed of this argument on summary judgment. The district court found that Teva and Dr. Reddy's had presented neither direct evidence of deceptive intent nor any evidence to support an inference of materiality. *SJ Enforceability Order* at 58. The strongest evidence of some problem was the passing comment of one Eisai "insider" that the similarity of lansoprazole and rabeprazole "bothers me." *Id.* at 59. But this vague, subjective statement is not sufficient by any means to establish materiality, let alone intent. Moreover, given lansoprazole's fluorinated substituent and its resultant impotence to render the '552 patent invalid, the district court properly rejected this strained theory of inequitable conduct on summary judgment.

IV

In a series of thoughtful, thorough opinions, the district court carefully explained its reasoning with respect to both obviousness and inequitable conduct. Because the district court properly concluded that Teva and Dr. Reddy's failed to prove that the '552 patent was invalid for obviousness or unenforceable for inequitable conduct, this court affirms the district court's judgment.

AFFIRMED

COSTS

Each party shall bear its own costs.

C.A.Fed. (N.Y.),2008.
Eisai Co. Ltd. v. Dr. Reddy's Laboratories, Ltd.
533 F.3d 1353, 87 U.S.P.Q.2d 1452

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EXHIBIT 4

SUPPLEMENTAL AMENDMENT TO MAY 4, 2009 AMENDMENT
FILED IN RESPONSE TO NOVEMBER 3, 2008 OFFICE ACTION

Submitted: October 7, 2009

Serial No. 10/821,726

Filed: April 8, 2004

Applicants: Michael Wayne Graham et al.

United States Court of Appeals,
Federal Circuit.
TAKEDA CHEMICAL INDUSTRIES, LTD. and Takeda Pharmaceuticals North America, INC.,
Plaintiffs-Appellees,
v.
ALPHAPHARM PTY., LTD. and Genpharm, Inc.,
Defendants-Appellants.
No. 06-1329.

June 28, 2007.

Background: Owner of patent for diabetes drug brought infringement actions against proposed manufacturers of generic versions. The United States District Court for the Southern District of New York, Denise Cote, J., 417 F.Supp.2d 341, granted judgment for owner. Manufacturers appealed.

Holdings: The Court of Appeals, Lourie, Circuit Judge, held that:
(1) person of ordinary skill in the art would not have selected closest prior art compound as lead compound for antidiabetic treatment;
(2) person of ordinary skill in the art would not have been prompted to modify closest prior art compound, using steps of homologation or ring-walking, to synthesize claimed compound; and
(3) any error was harmless that district court may have committed by incorrectly implying that prosecution histories were not accessible to public.

Affirmed.

Dyk, Circuit Judge, filed concurring opinion.

West Headnotes

[1] Patents 291 ⚿16.25

291 Patents
291II Patentability
291II(A) Invention; Obviousness
291k16.25 k. Chemical Compounds. Most Cited Cases
Person of ordinary skill in the art would not have selected closest prior art compound as lead compound for antidiabetic treatment, and thus presumption of motivation did not apply on competitor's claim of obviousness; although prosecution history of patent included statement characterizing compound as "especially important," any suggestion to select compound was essentially negated given more exhaustive and reliable scientific analysis which taught away from compound and evidence from similar contemporaneously filed patents showed that there were many promising, broad avenues for further research. 35 U.S.C.A. § 103.

[2] Patents 291 ⚿312(4)

291 Patents
291XII Infringement
291XII(C) Suits in Equity
291k312 Evidence
291k312(3) Weight and Sufficiency
291k312(4) k. Degree of Proof; Prima Facie Case. Most Cited Cases
Because a patent is presumed to be valid, the evidentiary burden to show facts supporting a conclusion of invalidity, which rests on the accused infringer, is one of clear and convincing evidence. 35 U.S.C.A. § 282.

[3] Patents 291 ⚿324.5

291 Patents
291XII Infringement
291XII(C) Suits in Equity
291k324 Appeal
291k324.5 k. Scope and Extent of Review in General. Most Cited Cases

Patents 291 ⚿324.55(4)

291 Patents
291XII Infringement
291XII(C) Suits in Equity
291k324 Appeal
291k324.55 Questions of Fact, Verdicts, and Findings
291k324.55(3) Issues of Validity
291k324.55(4) k. Novelty, Invention, Anticipation, and Obviousness. Most Cited Cases
Whether an invention would have been obvious is a question of law, reviewed de novo, based upon underlying factual questions which are reviewed for clear error following a bench trial. 35 U.S.C.A. § 103.

[4] Patents 291 ⚿16(2)

291 Patents
291II Patentability
291II(A) Invention; Obviousness
291k16 Invention and Obviousness in General
291k16(2) k. Prior Art in General. Most Cited Cases

Patents 291 ⚿16(3)

291 Patents
291II Patentability
291II(A) Invention; Obviousness
291k16 Invention and Obviousness in General
291k16(3) k. View of Person Skilled in Art. Most Cited Cases

Patents 291 ⚿36.1(1)

291 Patents
291II Patentability
291II(A) Invention; Obviousness
291k36 Weight and Sufficiency
291k36.1 Secondary Factors Affecting Invention or Obviousness
291k36.1(1) k. In General. Most Cited Cases
The factors that control an obviousness inquiry are: (1) the scope and content of the prior art; (2) the differences between the prior art and the claims; (3) the level of ordinary skill in the pertinent art; and (4) objective evidence of nonobviousness. 35 U.S.C.A. § 103.

[5] Patents 291 ⚿16.25

291 Patents
291II Patentability
291II(A) Invention; Obviousness
291k16.25 k. Chemical Compounds. Most Cited Cases
In a case involving a patent on a new chemical compound, some reason must be identified that would have led a chemist to modify a known compound in a particular manner to establish prima facie obviousness of a new claimed compound. 35 U.S.C.A. § 103.

[6] Patents 291 ⚿16.25

291 Patents
291II Patentability
291II(A) Invention; Obviousness
291k16.25 k. Chemical Compounds. Most Cited Cases
Person of ordinary skill in the art would not have been prompted to modify closest prior art compound, using steps of homologation or ring-walking, to synthesize claimed compound in patent for antidiabetic treatment, and thus claimed compound was not obvious, where process of modifying lead compounds was not routine at time of invention, nothing in prior art provided reasonable expectation that adding methyl group to compound would have reduced or eliminated toxicity of lead compound, there was no reasonable expectation in the art that changing positions of substituent on pyridyl ring would have resulted in beneficial

changes, and claimed compound differed significantly from lead compound, of which it was not a homolog, in terms of toxicity. 35 U.S.C.A. § 103.

[7] Patents 291 ⚿168(2.1)

291 Patents
291IX Construction and Operation of Letters Patent
291IX(B) Limitation of Claims
291k168 Proceedings in Patent Office in General
291k168(2) Rejection and Amendment of Claims
291k168(2.1) k. In General. Most Cited Cases

Statement made during prosecution of patent for antidiabetic treatment in response to enablement rejection, indicating only that changes to left moiety of lead compound would create compounds with same properties as compounds of prior art, did not represent that lower toxicity would result from change, for purpose of obviousness claim. 35 U.S.C.A. § 103.

[8] Patents 291 ⚿324.56

291 Patents
291XII Infringement
291XII(C) Suits in Equity
291k324 Appeal
291k324.56 k. Harmless Error. Most Cited Cases

Any error was harmless that district court may have committed by incorrectly implying that prosecution histories were not accessible to public, on competitor's claim of obviousness, where court nonetheless considered prosecution history of patent in its obviousness analysis and accorded proper weight to statements contained therein. 35 U.S.C.A. § 103.

Patents 291 ⚿328(2)

291 Patents
291XIII Decisions on the Validity, Construction, and Infringement of Particular Patents
291k328 Patents Enumerated
291k328(2) k. Original Utility. Most Cited Cases

4,287,200. Cited as Prior Art.

Patents 291 ⚿328(2)

291 Patents
291XIII Decisions on the Validity, Construction, and Infringement of Particular Patents
291k328 Patents Enumerated
291k328(2) k. Original Utility. Most Cited Cases

4,340,605, 4,438,141, 4,444,779. Cited.

Patents 291 ⚿328(2)

291 Patents
291XIII Decisions on the Validity, Construction, and Infringement of Particular Patents
291k328 Patents Enumerated
291k328(2) k. Original Utility. Most Cited Cases

4,687,777. Valid.

***1351** David G. Conlin, Edwards Angell Palmer & Dodge LLP, of Boston, MA, argued ***1352** for plaintiffs-appellees. With him on the brief were Barbara L. Moore, Kathleen B. Carr, and Adam P. Samansky; and Anthony J. Viola and Andre K. Cizmarik, of New York, NY. Of counsel on the brief was Mark Chao, Takeda Pharmaceuticals North America, Inc., of Lincolnshire, IL.

Kevin F. Murphy, Frommer Lawrence & Haug LLP, of New York, NY, argued for defendants-appellants. With him on the brief were Edgar H. Haug and Jeffrey A. Hovden.

Before LOURIE, BRYSON, and DYK, Circuit Judges.

Opinion for the court filed by Circuit Judge

LOURIE. Concurring opinion filed by Circuit Judge DYK.

LOURIE, Circuit Judge.

Alphapharm Pty., Ltd. and Genpharm, Inc. (collectively "Alphapharm") appeal from the decision of the United States District Court for the Southern District of New York, following a bench trial, that U.S. Patent 4,687,777 was not shown to be invalid under 35 U.S.C. § 103. *Takeda Chem. Indus., Ltd. v. Mylan Labs.*, 417 F.Supp.2d 341 (S.D.N.Y.2006). Because we conclude that the district court did not err in determining that the claimed compounds would not have been obvious in light of the prior art, and hence that the patent has not been shown to be invalid, we affirm.

BACKGROUND

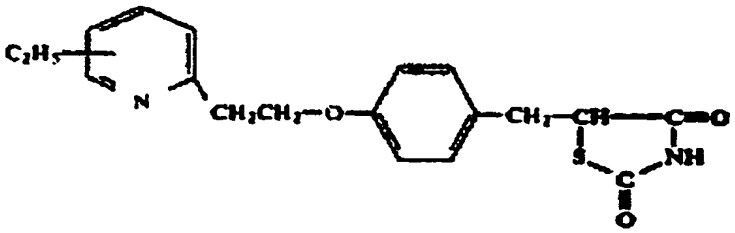
Diabetes is a disease that is characterized by the body's inability to regulate blood sugar. It is generally caused by inadequate levels of insulin-a hormone produced in the pancreas. Insulin allows blood sugar or glucose, which is derived from food, to enter into the body's cells and be converted into energy. There are two types of diabetes, known as Type 1 and Type 2. In Type 1 diabetes, the pancreas fails to produce insulin, and individuals suffering from this type of diabetes must regularly receive insulin from an external source. In contrast, Type 2 diabetic individuals produce insulin. However, their bodies are unable to effectively use the insulin that is produced. This is also referred to as insulin resistance. As a result, glucose is unable to enter the cells, thereby depriving the body of its main source of energy. Type 2 diabetes is the most common form of diabetes-affecting over 90% of diabetic individuals.

In the 1990s, a class of drugs known as thiazolidinediones ("TZDs") was introduced on the market as a treatment for Type 2 diabetes. Takeda Chemical Industries, Ltd., and Takeda Pharmaceuticals North America, Inc. (collectively "Takeda") first invented certain TZDs in the 1970s. Takeda's research revealed that TZDs acted as insulin sensitizers, *i.e.*, compounds that ameliorate insulin resistance. Although the function of TZDs was not completely understood, TZDs appeared to lower blood glucose levels by binding to a molecule in the nucleus of the cell known as PPARgamma, which activates insulin receptors and stimulates the production of glucose transporters. *Takeda*, 417 F.Supp.2d at 348-49. The transporters then travel to the cellular surface and enable glucose to enter the cell from the bloodstream. *Id.*

Takeda developed the drug ACTOS®, which is used to control blood sugar in patients who suffer from Type 2 diabetes. ACTOS® has enjoyed substantial commercial success since its launch in 1999. By ***1353** 2003, it held 47% of the TZD market, and gross sales for that year exceeded $1.7 billion. *Id.* at 386. The active ingredient in ACTOS® is the TZD compound pioglitazone, a compound claimed in the patent in suit.

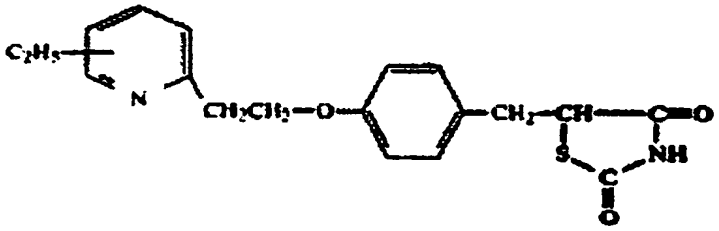
Takeda owns U.S. Patent 4,687,777 (the "'777 patent") entitled "Thiazolidinedione Derivatives, Useful As Antidiabetic Agents." The patent is directed to "compounds which can be practically used as antidiabetic agents having a broad safety margin between pharmacological effect and toxicity or unfavorable side reactions." '777 patent col.1 ll.34-37. The asserted claims are claims 1, 2, and 5. Claim 1 claims a genus of compounds. Claim 5 claims pharmaceutical compositions containing that genus of compounds. Those claims read as follows:

1. A compound of the formula:



or a pharmacologically acceptable salt thereof.

5. An antidiabetic composition which consists essentially of a compound of the formula:



or a pharmacologically acceptable salt thereof, in association with a pharmacologically acceptable carrier, excipient or diluent.
Id., claims 1 & 5.

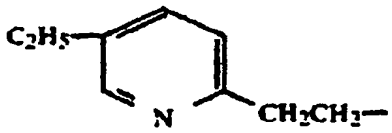
For purposes of this appeal, the critical portion of the compound structure is the left moiety of the molecule, namely, the ethyl-substituted pyridyl ring.[FN1] That chemical structure, which has an ethyl substituent (C^2 H^5) pictorially drawn to the center of the pyridyl ring, indicates that the structure covers four possible compounds, *viz.*, compounds with an ethyl substituent located at the four available positions on the pyridyl ring. *Takeda*, 417 F.Supp.2d at 360. The formula includes the 3-ethyl compound, 4-ethyl compound, 5-ethyl compound (pioglitazone), and 6-ethyl compound.

FN1. Pyridine is a "six-membered carbon-containing ring with one carbon replaced by a nitrogen." *Takeda*, 417 F.Supp.2d at 351.

***1354** Claim 2 of the '777 patent covers the single compound pioglitazone. That claim, which depends from claim 1, reads:

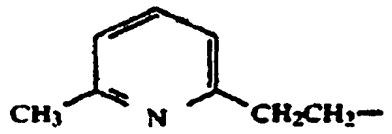
> 2. A compound as claimed in claim 1, wherein the compound is 5-{4-[2-(5-ethyl-2-pyridyl)ethoxy] benzyl}-2,4-thiazolidinedione.

'777 patent, claim 2. Pioglitazone is referred to as the 5-ethyl compound because the ethyl substituent is attached to the 5-position on the pyridyl ring. That portion of the compound is depicted as:



Alphapharm, a generic drug manufacturer, filed an Abbreviated New Drug Application ("ANDA") pursuant to the Hatch-Waxman Act seeking U.S. Food and Drug Administration ("FDA") approval under 21 U.S.C. § 355(j) et seq. to manufacture and sell a generic version of pioglitazone. Alphapharm filed a Paragraph IV certification with its ANDA pursuant to § 505(j)(2)(B)(ii), asserting that the '777 patent is invalid as obvious under 35 U.S.C. § 103. In response, Takeda sued Alphapharm, along with three other generic drug manufacturers who also sought FDA approval to market generic pioglitazone, alleging that the defendants have infringed or will infringe the '777 patent.

On January 17, 2006, the district court commenced a bench trial solely on the issues of validity and enforceability of the '777 patent. Alphapharm advanced its invalidity argument, asserting that the claimed compounds would have been obvious at the time of the alleged invention. Alphapharm's obviousness contention rested entirely on a prior art TZD compound that is referenced in Table 1 of the '777 patent as compound b. The left moiety of compound b consists of a pyridyl ring with a methyl (CH^3) group attached to the 6-position of the ring. That portion of its chemical structure is illustrated as follows:



Alphapharm asserted that the claimed compounds would have been obvious over compound b.

The district court found that Alphapharm failed to prove by clear and convincing evidence that the asserted claims were invalid as obvious under 35 U.S.C. § 103. The court first concluded that there was no motivation in the prior art to select compound b as the lead compound for antidiabetic research, and that the prior art taught away from its use. As such, the court concluded that Alphapharm failed to make a prima facie case of obviousness. The court continued its analysis and found that even if Alphapharm succeeded in making a prima facie showing, Takeda would still prevail because any prima facie case of obviousness was rebutted by the unexpected results of pioglitazone's nontoxicity. The court then rendered judgment in favor of Takeda. The district court also held that the '777 patent had not been procured though inequitable conduct. That decision has been separately appealed and has been affirmed in a decision issued today.

Alphapharm timely appealed. We have jurisdiction pursuant to 28 U.S.C. § 1295(a)(1).

DISCUSSION

A. Standard of Review

[1][2][3] In this appeal, we are presented with one

issue, namely, whether the asserted***1355** claims of the '777 patent would have been obvious under 35 U.S.C. § 103 at the time the invention was made. An invention is not patentable, *inter alia,* "if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art." 35 U.S.C. § 103(a). Because a patent is presumed to be valid, 35 U.S.C. § 282, the evidentiary burden to show facts supporting a conclusion of invalidity, which rests on the accused infringer, is one of clear and convincing evidence. *AK Steel Corp. v. Sollac & Ugine,* 344 F.3d 1234, 1238-39 (Fed.Cir.2003). Whether an invention would have been obvious under 35 U.S.C. § 103 is a "question of law, reviewed de novo, based upon underlying factual questions which are reviewed for clear error following a bench trial." *Alza Corp. v. Mylan Labs., Inc.,* 464 F.3d 1286, 1289 (Fed.Cir.2006).

B. Obviousness

Alphapharm raises three main arguments in support of its contention that the claims would have been obvious. First, Alphapharm asserts that the district court misapplied the law, particularly the law governing obviousness in the context of structurally similar chemical compounds. According to Alphapharm, the record established that compound b was the most effective antidiabetic compound in the prior art, and thus the court erred by failing to apply a presumption that one of ordinary skill in the art would have been motivated to make the claimed compounds. Alphapharm asserts that such a conclusion is mandated by our case law, including our en banc decision in *In re Dillon,* 919 F.2d 688 (Fed.Cir.1990). Second, Alphapharm argues that the court erred in determining the scope and content of the prior art, in particular, whether to include the prosecution history of the prior '779 patent. Lastly, Alphapharm assigns error to numerous legal and factual determinations and certain evidentiary rulings that the court made during the course of the trial.

Takeda responds that the district court correctly determined that Alphapharm failed to prove by clear and convincing evidence that the asserted claims are invalid as obvious. Takeda contends that there was overwhelming evidence presented at trial to support the court's conclusion that no motivation existed in the prior art for one of ordinary skill in the art to select compound b as a lead compound, and even if there was, that the unexpected results of pioglitazone's improved toxicity would have rebutted any prima facie showing of obviousness. Takeda further argues that all of Alphapharm's remaining challenges to the district court's legal and factual rulings are simply without merit.

[4] We agree with Takeda that the district court did not err in concluding that the asserted claims of the '777 patent would not have been obvious. The Supreme Court recently addressed the issue of obviousness in *KSR International Co. v. Teleflex Inc.,* 550 U.S. 398, 127 S.Ct. 1727, 167 L.Ed.2d 705 (2007). The Court stated that the *Graham v. John Deere Co. of Kansas City,* 383 U.S. 1, 86 S.Ct. 684, 15 L.Ed.2d 545 (1966), factors still control an obviousness inquiry. Those factors are: 1) "the scope and content of the prior art"; 2) the "differences between the prior art and the claims"; 3) "the level of ordinary skill in the pertinent art"; and 4) objective evidence of nonobviousness. *KSR,* 127 S.Ct. at 1734 (quoting *Graham,* 383 U.S. at 17-18, 86 S.Ct. 684).

In a thorough and well-reasoned opinion, albeit rendered before *KSR* was decided ***1356** by the Supreme Court, the district court made extensive findings of fact and conclusions of law as to the four *Graham* factors. Alphapharm's arguments challenge the court's determinations with respect to certain of these factors, which we now address.

1. Differences Between the Prior Art and the Claims

a. Selection of Compound b as Lead Compound

Alphapharm's first argument challenges the court's determination with regard to the "differences between the prior art and the claims." Alphapharm contends that the court erred as a matter of law in holding that the ethyl-substituted TZDs were nonobvious in light of the closest prior art compound, compound b, by misapplying the law relating to obviousness of chemical compounds.

We disagree. Our case law concerning prima facie obviousness of structurally similar compounds is well-established. We have held that "structural similarity between claimed and prior art subject matter, proved by combining references or otherwise, where the prior art gives reason or motivation to make the claimed compositions, creates a prima facie case of obviousness." *Dillon*, 919 F.2d at 692. In addition to structural similarity between the compounds, a prima facie case of obviousness also requires a showing of "adequate support in the prior art" for the change in structure. *In re Grabiak*, 769 F.2d 729, 731-32 (Fed.Cir.1985).

We elaborated on this requirement in the case of *In re Deuel*, 51 F.3d 1552, 1558 (Fed.Cir.1995), where we stated that "[n]ormally a prima facie case of obviousness is based upon structural similarity, *i.e.*, an established structural relationship between a prior art compound and the claimed compound." That is so because close or established "[s]tructural relationships may provide the requisite motivation or suggestion to modify known compounds to obtain new compounds." *Id.* A known compound may suggest its homolog, analog, or isomer because such compounds "often have similar properties and therefore chemists of ordinary skill would ordinarily contemplate making them to try to obtain compounds with improved properties." *Id.* We clarified, however, that in order to find a prima facie case of unpatentability in such instances, a showing that the "prior art would have suggested making the specific molecular modifications necessary to achieve the claimed invention" was also required. *Id.* (citing *In re Jones*, 958 F.2d 347 (Fed.Cir.1992); *Dillon*, 919 F.2d 688; *Grabiak*, 769 F.2d 729; *In re Lalu*, 747 F.2d 703 (Fed.Cir.1984)).

[5] That test for prima facie obviousness for chemical compounds is consistent with the legal principles enunciated in *KSR*.[FN2] While the *KSR* Court rejected a rigid application of the teaching, suggestion, or motivation ("TSM") test in an obviousness inquiry, the Court acknowledged the importance of identifying "a reason that would have prompted a person of ordinary skill in the relevant field to combine***1357** the elements in the way the claimed new invention does" in an obviousness determination. *KSR*, 127 S.Ct. at 1731. Moreover, the Court indicated that there is "no necessary inconsistency between the idea underlying the TSM test and the *Graham* analysis." *Id.* As long as the test is not applied as a "rigid and mandatory" formula, that test can provide "helpful insight" to an obviousness inquiry. *Id.* Thus, in cases involving new chemical compounds, it remains necessary to identify some reason that would have led a chemist to modify a known compound in a particular manner to establish prima facie obviousness of a new claimed compound.

> FN2. We note that the Supreme Court in its *KSR* opinion referred to the issue as whether claimed subject matter "was" or "was not" obvious. Since 35 U.S.C. § 103 uses the language "would have been obvious," and the Supreme Court in *KSR* did consider the particular time at which obviousness is determined, we consider that the Court did not in *KSR* reject the standard statutory formulation of the inquiry whether the claimed subject matter "would have been obvious at the time the invention was

made." 35 U.S.C. § 103. Hence, we will continue to use the statutory "would have been" language.

We agree with Takeda and the district court that Alphapharm failed to make that showing here. Alphapharm argues that the prior art would have led one of ordinary skill in the art to select compound b as a lead compound. By "lead compound," we understand Alphapharm to refer to a compound in the prior art that would be most promising to modify in order to improve upon its antidiabetic activity and obtain a compound with better activity.[FN3] Upon selecting that compound for antidiabetic research, Alphapharm asserts that one of ordinary skill in the art would have made two obvious chemical changes: first, homologation, *i.e.*, replacing the methyl group with an ethyl group, which would have resulted in a 6-ethyl compound; and second, "ring-walking," or moving the ethyl substituent to another position on the ring, the 5-position, thereby leading to the discovery of pioglitazone. Thus, Alphapharm's obviousness argument clearly depends on a preliminary finding that one of ordinary skill in the art would have selected compound b as a lead compound.

FN3. The parties do not dispute that compound b was the closest prior art compound. Thus, the legal question is whether or not the claimed subject matter would have been obvious over that compound. We will, however, use Alphapharm's terminology of "lead compound" in this opinion, deciding the appeal as it has been argued.

The district court found, however, that one of ordinary skill in the art would not have selected compound b as the lead compound. In reaching its determination, the court first considered Takeda's U.S. Patent 4,287,200 (the "'200 patent"), which was issued on September 1, 1981, and its prosecution history. The court found that the '200 patent discloses hundreds of millions of TZD compounds."[FN4] *Takeda*, 417 F.Supp.2d at 378. The patent specifically identified fifty-four compounds, including compound b, that were synthesized according to the procedures described in the patent, but did not disclose experimental data or test results for any of those compounds. The prosecution history, however, disclosed test results for nine specific compounds, including compound b. That information was provided to the examiner in response to a rejection in order to show that the claimed compounds of the ' 200 patent were superior to the known compounds that were disclosed in a cited reference. The court, however, found nothing in the '200 patent, or in its file history, to suggest to one of ordinary skill in the art that those nine compounds, out of the hundreds of millions of compounds covered by the patent application, were the best performing compounds as antidiabetics, and hence targets for modification to seek improved properties. *Id.* at 375.

FN4. Three divisional applications derive from the '200 patent. Those applications matured into U.S. Patent 4,340,605, U.S. Patent 4,438,141, and U.S. Patent No. 4,444,779 (the "'779 Patent"). The ' 779 patent is of particular relevance in this appeal and is discussed below. *Takeda*, 417 F.Supp.2d at 378.

***1358** The court next considered an article that was published the following year in 1982 by T. Sodha et al. entitled "Studies on Antidiabetic Agents. II. Synthesis of 5-[4-(1-Methylcyclohexylmethoxy)-benzyl]thiazolidine-2,4-dione (ADD-3878) and Its Derivatives" ("Sodha II"). The Sodha II reference disclosed data relating to hypoglycemic activity and plasma triglyceride lowering activity for 101 TZD compounds. Those compounds did not include pioglitazone, but included compound b. Significantly, Sodha II identified three specific compounds that were deemed most favorable in terms of toxicity and activity.

Notably, compound b was not identified as one of the three most favorable compounds. On the contrary, compound b, was singled out as causing "considerable increases in body weight and brown fat weight."

The court also considered Takeda's '779 patent. That patent covers a subset of compounds originally included in the '200 patent application, namely, TZD compounds "where the pyridyl or thiazolyl groups may be substituted." *Id.* at 353. The broadest claim of the '779 patent covers over one million compounds. *Id.* at 378. Compound b was specifically claimed in claim 4 of the patent. The court noted that a preliminary amendment in the prosecution history of the patent contained a statement that "the compounds in which these heterocyclic rings are substituted have become important, especially [compound b]." *Id.*

Based on the prior art as a whole, however, the court found that a person of ordinary skill in the art would not have selected compound b as a lead compound for antidiabetic treatment. Although the prosecution history of the '779 patent included the statement that characterized compound b as "especially important," the court found that any suggestion to select compound b was essentially negated by the disclosure of the Sodha II reference. The court reasoned that one of ordinary skill in the art would not have chosen compound b, notwithstanding the statement in the '779 patent prosecution history, "given the more exhaustive and reliable scientific analysis presented by Sodha II, which taught away from compound b, and the evidence from all of the TZD patents that Takeda filed contemporaneously with the '779 [p]atent showing that there were many promising, broad avenues for further research." *Id.* at 380.

The court found that the three compounds that the Sodha II reference identified as "most favorable" and "valuable for the treatment of maturity-onset diabetes," not compound b, would have served as the best "starting point for further investigation" to a person of ordinary skill in the art. *Id.* at 376. Because diabetes is a chronic disease and thus would require long term treatment, the court reasoned that researchers would have been dissuaded from selecting a lead compound that exhibited negative effects, such as toxicity, or other adverse side effects, especially one that causes "considerable increases in body weight and brown fat weight." *Id.* at 376-77. Thus, the court determined that the prior art did not suggest to one of ordinary skill in the art that compound b would be the best candidate as the lead compound for antidiabetic research.

Admissions from Alphapharm witnesses further buttressed the court's conclusion. Dr. Rosenberg, head of Alphapharm's intellectual property department, testified as a 30(b)(6) witness on behalf of Alphapharm. In discussing Sodha II, Dr. Rosenberg admitted that there was nothing in ***1359** the article that would recommend that a person of ordinary skill in the art choose compound b over other compounds in the article that had the same efficacy rating. Dr. Rosenberg, acknowledging that compound b had the negative side effects of increased body weight and brown fat, also admitted that a compound with such side effects would "presumably not" be a suitable candidate compound for treatment of Type II diabetes. Alphapharm's expert, Dr. Mosberg, concurred in that view at his deposition when he admitted that a medicinal chemist would find such side effects "undesirable."

Moreover, another Alphapharm 30(b)(6) witness, Barry Spencer, testified at his deposition that in reviewing the prior art, one of ordinary skill in the art would have chosen three compounds in Sodha II as lead compounds for research, not solely compound b. In addition, Takeda's witness, Dr. Morton, testified that at the time Sodha II was published, it was known that obesity contributed to insulin resistance and Type 2 diabetes. Thus, one of ordinary skill in the art would have concluded that Sodha II taught away from pyridyl compounds because it associated

adverse side effects with compound b.

We do not accept Alphapharm's assertion that *KSR*, as well as another case recently decided by this court, *Pfizer, Inc. v. Apotex, Inc.*, 480 F.3d 1348 (Fed.Cir.2007), mandates reversal. Relying on *KSR*, Alphapharm argues that the claimed compounds would have been obvious because the prior art compound fell within "the objective reach of the claim," and the evidence demonstrated that using the techniques of homologation and ring-walking would have been "obvious to try." Additionally, Alphapharm argues that our holding in *Pfizer*, where we found obvious certain claims covering a particular acid-addition salt, directly supports its position.

We disagree. The *KSR* Court recognized that "[w]hen there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp." *KSR*, 127 S.Ct. at 1732. In such circumstances, "the fact that a combination was obvious to try might show that it was obvious under § 103." *Id.* That is not the case here. Rather than identify predictable solutions for antidiabetic treatment, the prior art disclosed a broad selection of compounds any one of which could have been selected as a lead compound for further investigation. Significantly, the closest prior art compound (compound b, the 6-methyl) exhibited negative properties that would have directed one of ordinary skill in the art away from that compound. Thus, this case fails to present the type of situation contemplated by the Court when it stated that an invention may be deemed obvious if it was "obvious to try." The evidence showed that it was not obvious to try.

Similarly, Alphapharm's reliance on *Pfizer* fares no better. In *Pfizer*, we held that certain claims covering the besylate salt of amlodipine would have been obvious. The prior art included a reference, referred to as the Berge reference, that disclosed a genus of pharmaceutically acceptable anions that could be used to form pharmaceutically acceptable acid addition salts, as well as other publications that disclosed the chemical characteristics of the besylate salt. *Pfizer*, 480 F.3d at 1363. Noting that our conclusion was based on the "particularized facts of this case," we found that the prior art provided ***1360** "ample motivation to narrow the genus of 53 pharmaceutically-acceptable anions disclosed by Berge to a few, including benzene sulphonate." *Id.* at 1363, 1367. Here, the court found nothing in the prior art to narrow the possibilities of a lead compound to compound b. In contrast, the court found that one of ordinary skill in the art would have chosen one of the many compounds disclosed in Sodha II, of which there were over ninety, that "did not disclose the existence of toxicity or side effects, and to engage in research to increase the efficacy and confirm the absence of toxicity of those compounds, rather than to choose as a starting point a compound with identified adverse effects." Thus, *Pfizer* does not control this case.

Based on the record before us, we conclude that the district court's fact-findings were not clearly erroneous and were supported by evidence in the record. Moreover, we reject the assertion that the court failed to correctly apply the law relating to prima facie obviousness of chemical compounds. Because Alphapharm's obviousness argument rested entirely on the court making a preliminary finding that the prior art would have led to the selection of compound b as the lead compound, and Alphapharm failed to prove that assertion, the court did not commit reversible error by failing to apply a presumption of motivation. We thus conclude that the court did not err in holding that Alphapharm failed to establish a prima facie case of obviousness. *See Eli Lilly & Co. v. Zenith Goldline Pharms.*, 471 F.3d 1369 (Fed.Cir.2006) (affirming the district court's finding of nonobviousness upon concluding, in part, that the prior art compound would not have been chosen as a lead compound).

b. Choice of the Claimed Compounds

[6] Even if Alphapharm had established that preliminary finding, and we have concluded that it did not, the record demonstrates that Alphapharm's obviousness argument fails on a second ground. The district court found nothing in the prior art to suggest making the specific molecular modifications to compound b that are necessary to achieve the claimed compounds. In reaching that conclusion, the court first found that the process of modifying lead compounds was not routine at the time of the invention. *Takeda.* 417 F.Supp.2d at 380. Dr. Mosberg opined that the steps of homologation and ring-walking were "routine steps in the drug optimization process," but the court found that testimony unavailing in light of the contrary, more credible, testimony offered by Takeda's experts. *Id.* at 381. In addition, the court relied on Dr. Rosenberg's admission that a person of ordinary skill in the art would "look at a host of substituents, such as chlorides, halides and others, not just methyls" in modifying the pyridyl ring. *Id.*

Pioglitazone differs from compound b in two respects, and one would have to both homologate the methyl group of compound b and move the resulting ethyl group to the 5-position on the pyridyl ring in order to obtain pioglitazone. With regard to homologation, the court found nothing in the prior art to provide a reasonable expectation that adding a methyl group to compound b would reduce or eliminate its toxicity. Based on the test results of the numerous compounds disclosed in Sodha II, the court concluded that "homologation had no tendency to decrease unwanted side effects" and thus researchers would have been inclined "to focus research efforts elsewhere." *Id.* at 383. Indeed, several other compounds exhibited similar or better potency than compound b, and one compound in particular, compound 99, that had no identified problems differed significantly***1361** from compound b in structure. *Id.* at 376 n. 51. Moreover, Dr. Mosberg agreed with Takeda's expert, Dr. Danishefsky, that the biological activities of various substituents were "unpredictable" based on the disclosure of Sodha II. *Id.* at 384-85. The court also found nothing in the '200 and '779 patents to suggest to one of ordinary skill in the art that homologation would bring about a reasonable expectation of success.

As for ring-walking, the court found that there was no reasonable expectation in the art that changing the positions of a substituent on a pyridyl ring would result in beneficial changes. Dr. Mosberg opined that the process of ring-walking was "known" to Takeda, but the court found that testimony inapt as it failed to support a reasonable expectation to one of ordinary skill in the art that performing that chemical change would cause a compound to be more efficacious or less toxic. *Id.* at 382. Moreover, Dr. Mosberg relied on the efficacy data of phenyl compounds in Sodha II, but the court found those data insufficient to show that the same effects would occur in pyridyl compounds.

Alphapharm relies on *In re Wilder,* 563 F.2d 457 (CCPA 1977), for the proposition that differences in a chemical compound's properties, resulting from a small change made to the molecule, are reasonably expected to vary by degree and thus are insufficient to rebut a prima facie case of obviousness. In *Wilder,* our predecessor court affirmed the Board's holding that a claimed compound, which was discovered to be useful as a rubber antidegradant and was also shown to be nontoxic to human skin, would have been obvious in light of its homolog and isomer that were disclosed in the prior art. The evidence showed that the homolog was similarly nontoxic to the human skin, whereas the isomer was toxic. The court held that "one who claims a compound, per se, which is structurally similar to a prior art compound must rebut the presumed expectation that the structurally similar compounds have similar properties." *Id.* at 460. While recognizing that the difference between the isomer's toxicity and the nontoxicity of the homolog and claimed compound "indicate[d] some degree of un-

predictability," the court found that the appellant failed to "point out a single actual difference in properties between the claimed compound and the homologue," and thus failed to rebut the presumption. *Wilder,* 563 F.2d at 460.

We would note that since our *Wilder* decision, we have cautioned "that generalization should be avoided insofar as specific chemical structures are alleged to be prima facie obvious one from the other," *Grabiak,* 769 F.2d at 731. In addition to this caution, the facts of the present case differ significantly from the facts of Wilder. Here, the court found that pioglitazone exhibited unexpectedly superior properties over the prior art compound b. *Takeda,* 417 F.Supp.2d at 385. The court considered a report entitled "Preliminary Studies on Toxicological Effects of Ciglitazone-Related Compounds in the Rats" that was presented in February 1984 by Dr. Takeshi Fujita, then-Chief Scientist of Takeda's Biology Research Lab and co-inventor of the '777 patent. That report contained results of preliminary toxicity studies that involved selected compounds, including pioglitazone and compound b. Compound b was shown to be "toxic to the liver, heart and erythrocytes, among other things," whereas pioglitazone was "comparatively potent" and "showed no statistically significant toxicity." *Id.* at 356-57. During the following months, Takeda performed***1362** additional toxicity studies on fifty compounds that had been already synthesized and researched by Takeda, including pioglitazone. The compounds were tested for potency and toxicity. The results were presented in another report by Fujita entitled "Pharmacological and Toxicological Studies of Ciglitazone and Its Analogues." Pioglitazone was shown to be the only compound that exhibited no toxicity, although many of the other compounds were found to be more potent. *Id.* at 358.

Thus, the court found that there was no reasonable expectation that pioglitazone would possess the desirable property of nontoxicity, particularly in light of the toxicity of compound b. The court's characterization of pioglitazone's unexpected results is not clearly erroneous. As such, *Wilder* does not aid Alphapharm because, unlike the homolog and claimed compound in *Wilder* that shared similar properties, pioglitazone was shown to differ significantly from compound b, of which it was not a homolog, in terms of toxicity. Consequently, Takeda rebutted any presumed expectation that compound b and pioglitazone would share similar properties.

[7] Alphapharm also points to a statement Takeda made during the prosecution of the '779 patent as evidence that there was a reasonable expectation that making changes to the pyridyl region of compound b would lead to "better toxicity than the prior art." During prosecution of the '779 patent, in response to an enablement rejection, Takeda stated that "there should be no reason in the instant case for the Examiner to doubt that the claimed compounds having the specified substituent would function as a hypolipidemic and hypoglycemic agent as specified in the instant disclosure." That statement, however, indicates only that changes to the left moiety of a lead compound would create compounds with the same properties as the compounds of the prior art; it does not represent that lower toxicity would result. And even if the statement did so represent, it does not refer to any specific substituent at any specific position of TZD's left moiety as particularly promising. As the court correctly noted, the compounds disclosed in the ' 779 patent included a variety of substituents, including lower alkyls, halogens, and hydroxyl groups, attached to a pyridyl or thiazolyl group. As discussed *supra,* the district court found that the claims encompassed over one million compounds. Thus, we disagree with Alphapharm that that statement provided a reasonable expectation to one of ordinary skill in the art that performing the specific steps of replacing the methyl group of the 6-methyl compound with an ethyl group, and moving that substituent to the 5-position of the ring, would have

provided a broad safety margin, particularly in light of the district court's substantiated findings to the contrary.

We thus conclude that Alphapharm's challenges fail to identify grounds for reversible error. The court properly considered the teachings of the prior art and made credibility determinations regarding the witnesses at trial. We do not see any error in the district court's determination that one of ordinary skill in the art would not have been prompted to modify compound b, using the steps of homologation and ring-walking, to synthesize the claimed compounds. Because the court's conclusions are not clearly erroneous and are supported by the record evidence, we find no basis to disturb them.

The court properly concluded that Alphapharm did not make out a prima facie case of obviousness because Alphapharm ***1363** failed to adduce evidence that compound b would have been selected as the lead compound and, even if that preliminary showing had been made, it failed to show that there existed a reason, based on what was known at the time of the invention, to perform the chemical modifications necessary to achieve the claimed compounds.

In light of our conclusion that Alphapharm failed to prove that the claimed compounds would have been prima facie obvious, we need not consider any objective indicia of nonobviousness.[FN5]

> FN5. The concurrence, while agreeing that the question of the "overbreadth" of claims 1 and 5 has been waived, states further that the 6-ethyl compound, which is within the scope of claims 1 and 5, has not been shown to possess unexpected results sufficient to overcome a prima facie case of obviousness, and hence claims 1 and 5 are likely invalid as obvious. Since waiver is sufficient to answer the point being raised, no further comment need be made concerning its substance.

2. Scope and Content of the Prior Art

[8] Alphapharm also assigns error to the district court's determination regarding the scope and content of the prior art. Alphapharm asserts that the court excluded the prosecution history of the '779 patent from the scope of the prior art after wrongly concluding that it was not accessible to the public. Takeda responds that the court clearly considered the '779 patent prosecution history, which was admitted into evidence on the first day of testimony. Takeda urges that the court's consideration of the prosecution history is apparent based on its extensive analysis of the '779 patent and the file history that appears in the court's opinion.

We agree with Takeda that the district court did not err in its consideration of the scope of the prior art. As discussed above, the court considered the prosecution history, and even expressly considered one of the key statements in the prosecution history upon which Alphapharm relies in support of its position that compound b would have been chosen as the lead compound. *Takeda*, 417 F.Supp.2d at 378. In considering the prosecution history of the '779 patent, the court noted that Takeda filed a preliminary amendment on March 15, 1983, in which its prosecuting attorney stated that "the compounds in which these heterocyclic rings are substituted have become important, especially [the 6-methyl compound]." *Id.* The court rejected Alphapharm's assertion that that statement supported the conclusion that compound b would have been selected as a lead compound. Rather, the court found that viewing the prior art as a whole, the prior art showed "that Takeda was actively conducting research in many directions, and had not narrowed its focus to compound b." *Id.* at 379. Thus, while the district court may have incorrectly implied that prosecution histories are not accessible to the public, *see id.* at n. 59, *see also Custom Accessories, Inc. v. Jeffrey-Allan Indus.*, 807 F.2d 955 (Fed.Cir.1986) ("[t]he person of ordinary skill is a hypothetical person who is presumed to be aware of all the pertinent

prior art"), the court nonetheless considered the prosecution history of the '779 patent in its obviousness analysis and accorded proper weight to the statements contained therein. Thus, any error committed by the court in this regard was harmless error.

We have considered Alphapharm's remaining arguments and find none that warrant reversal of the district court's decision.

*1364 CONCLUSION

We affirm the district court's determination that claims 1, 2, and 5 of the ' 777 patent have not been shown to have been obvious and hence invalid.

AFFIRMED

Concurring opinion filed by Circuit Judge DYK.DYK, Circuit Judge, concurring.
I join the opinion of the court insofar as it upholds the district court judgment based on a determination that a claim to pioglitazone (the 5-ethyl compound) would be non-obvious over the prior art. The problem is that only one of the three claims involved here-claim 2-is limited to pioglitazone. In my view, the breadth of the other two claims, claims 1 and 5 of U.S. Patent No. 4,867,777 ("'777 patent")-which are also referenced in the judgment-renders them likely invalid.

All of the compounds claimed in claims 1, 2 and 5 were included in generic claims in the prior art U.S. Patent No. 4,287,200 ("'200 patent"). Unfortunately our law concerning when a species is patentable over a genus claimed in the prior art is less than clear. It is, of course, well established that a claim to a genus does not necessarily render invalid a later claim to a species within that genus. *See Eli Lilly & Co. v. Bd. of Regents of Univ. of Wash.*, 334 F.3d 1264, 1270 (Fed.Cir.2003). In my view a species should be patentable over a genus claimed in the prior art only if unexpected results have been established. Our case law recognizes the vital importance of a finding of unexpected results, both in this context and in the closely related context where a prior art patent discloses a numerical range and the patentee seeks to claim a subset of that range. *See Application of Petering*, 49 C.C.P.A. 993, 301 F.2d 676, 683 (1962) (species found patentable when genus claimed in prior art because unexpected properties of the species were shown); *see also Pfizer, Inc. v. Apotex, Inc.*, 480 F.3d 1348, 1371 (Fed.Cir.2007) (relying on lack of unexpected results in determining that species claim was obvious in view of prior art genus claim); *In re Woodruff*, 919 F.2d 1575, 1578 (Fed.Cir.1990) (when applicant claims a subset of a range disclosed in a prior art patent, the applicant must generally show that "the claimed range achieves unexpected results relative to the prior art range.").

While the 5-ethyl compound (pioglitazone) is within the scope of the '200 patent, there is clear evidence, as the majority correctly finds, of unexpected results regarding that compound, and therefore its validity is not in question on this ground. However, at oral argument the patentee admitted that the prior art '200 patent also generically covers the 6-ethyl compound, which is within the scope of claims 1 and 5 of the '777 patent, and admitted that there is no evidence of unexpected results for the 6-ethyl compound. Under such circumstances, I believe that the 6-ethyl is likely obvious, and consequently claims 1 and 5 are likely invalid for obviousness. However, the argument as to the overbreadth of claims 1 and 5 has been waived, because it was not raised in the opening brief. In any event, as a practical matter, the judgment finding that the appellants' filing of the ANDA for pioglitazone is an infringement and barring the making of pioglitazone is supported by the finding that claim 2 standing alone is not invalid and is infringed.

C.A.Fed. (N.Y.),2007.
Takeda Chemical Industries, Ltd. v. Alphapharm Pty., Ltd.

492 F.3d 1350, 83 U.S.P.Q.2d 1169

END OF DOCUMENT

EXHIBIT 5

SUPPLEMENTAL AMENDMENT TO MAY 4, 2009 AMENDMENT
FILED IN RESPONSE TO NOVEMBER 3, 2008 OFFICE ACTION

Submitted: October 7, 2009

Serial No. 10/821,726

Filed: April 8, 2004

Applicants: Michael Wayne Graham et al.

441 F.3d 991, 78 U.S.P.Q.2d 1417
(Cite as: 441 F.3d 991)

▷

United States Court of Appeals,
Federal Circuit.
ATOFINA, Plaintiff-Appellant,
v.
GREAT LAKES CHEMICAL CORPORATION,
Defendant-Appellee.
No. 05-1359.

March 23, 2006.
Rehearing and Rehearing En Banc Denied May 16, 2006.

Background: Owner of patent for method of synthesizing difluoromethane (CH_2F_2) sued competitor for infringement. The United States District Court for the District of Delaware, Sue L. Robinson, Chief Judge, 2005 WL 984361, found patent not infringed, invalid, and unenforceable. Owner appealed.

Holdings: The Court of Appeals, Lourie, Circuit Judge, held that:
(1) patent was not infringed;
(2) patent was not anticipated; and
(3) owner did not engage in inequitable conduct before Patent and Trademark Office.

Affirmed in part and reversed in part.

Dyk, Circuit Judge, concurred in part, dissented in part, and filed opinion.

West Headnotes

[1] Patents 291 ⇐324.5

291 Patents
291XII Infringement
291XII(C) Suits in Equity
291k324 Appeal
291k324.5 k. Scope and Extent of Review in General. Most Cited Cases
Patent claim construction is issue of law, reviewed de novo.

[2] Patents 291 ⇐324.55(5)

291 Patents
291XII Infringement
291XII(C) Suits in Equity
291k324 Appeal
291k324.55 Questions of Fact, Verdicts, and Findings
291k324.55(5) k. Issues of Infringement. Most Cited Cases
Finding of patent infringement is question of fact, reviewed for clear error.

[3] Patents 291 ⇐324.55(4)

291 Patents
291XII Infringement
291XII(C) Suits in Equity
291k324 Appeal
291k324.55 Questions of Fact, Verdicts, and Findings
291k324.55(3) Issues of Validity
291k324.55(4) k. Novelty, Invention, Anticipation, and Obviousness. Most Cited Cases
Patent anticipation is question of fact, reviewed for clear error. 35 U.S.C.A. § 102.

[4] Patents 291 ⇐324.54

291 Patents
291XII Infringement
291XII(C) Suits in Equity
291k324 Appeal
291k324.54 k. Presumptions and Discretion of Lower Court. Most Cited Cases

Patents 291 ⇐324.55(2)

291 Patents
291XII Infringement
291XII(C) Suits in Equity

291k324 Appeal
291k324.55 Questions of Fact, Verdicts, and Findings
291k324.55(2) k. Clearly Erroneous Findings. Most Cited Cases
District court's ultimate determination of inequitable conduct, rendering patent unenforceable, is reviewed for abuse of discretion, while its threshold findings regarding materiality and intent to mislead are reviewed for clear error.

[5] Patents 291 ⚿165(1)

291 Patents
291IX Construction and Operation of Letters Patent
291IX(B) Limitation of Claims
291k165 Operation and Effect of Claims in General
291k165(1) k. In General. Most Cited Cases

Patents 291 ⚿167(1)

291 Patents
291IX Construction and Operation of Letters Patent
291IX(B) Limitation of Claims
291k167 Specifications, Drawings, and Models
291k167(1) k. In General. Most Cited Cases

Patents 291 ⚿168(2.1)

291 Patents
291IX Construction and Operation of Letters Patent
291IX(B) Limitation of Claims
291k168 Proceedings in Patent Office in General
291k168(2) Rejection and Amendment of Claims
291k168(2.1) k. In General. Most Cited Cases
Court's primary focus in determining the ordinary and customary meaning of patent claim limitation is to consider intrinsic evidence of record, viz., patent itself, including claims, specification and, if in evidence, prosecution history, from perspective of one of ordinary skill in the art.

[6] Patents 291 ⚿159

291 Patents
291IX Construction and Operation of Letters Patent
291IX(A) In General
291k159 k. Extrinsic Evidence in General. Most Cited Cases
Although patent-construing court may rely on dictionary definitions of claim terms, court must ensure that any reliance on dictionaries accords with intrinsic evidence: claims themselves, specification, and prosecution history.

[7] Patents 291 ⚿101(2)

291 Patents
291IV Applications and Proceedings Thereon
291k101 Claims
291k101(2) k. Construction in General. Most Cited Cases
"Chromium catalyst," called for in patent for method of synthesizing difluoromethane (CH_2F_2), was catalyst in which only catalytically active material was chromium without addition of metal oxides or noninert additives.

[8] Patents 291 ⚿167(1.1)

291 Patents
291IX Construction and Operation of Letters Patent
291IX(B) Limitation of Claims
291k167 Specifications, Drawings, and Models
291k167(1.1) k. Specification as Limiting or Enlarging Claims. Most Cited Cases

Patents 291 ⚿168(2.1)

291 Patents
291IX Construction and Operation of Letters Patent
291IX(B) Limitation of Claims

291k168 Proceedings in Patent Office in General
291k168(2) Rejection and Amendment of Claims
291k168(2.1) k. In General. Most Cited Cases
Meaning of patent claim language may be limited by disclaimer in specification or prosecution history.

[9] Patents 291 ⚿168(2.1)

291 Patents
291IX Construction and Operation of Letters Patent
291IX(B) Limitation of Claims
291k168 Proceedings in Patent Office in General
291k168(2) Rejection and Amendment of Claims
291k168(2.1) k. In General. Most Cited Cases
Patentee is held to amount of claim scope surrendered by amendment, even if amendment surrendered more than may have been absolutely necessary to avoid particular prior art.

[10] Patents 291 ⚿229

291 Patents
291XII Infringement
291XII(A) What Constitutes Infringement
291k228 Patents for Processes
291k229 k. Identity in General. Most Cited Cases
Patent for method of synthesizing difluoromethane (CH_2F_2), calling for use of pure chromium catalyst, was not infringed by accused process whose chromium catalyst included non-inert additives.

[11] Patents 291 ⚿72(1)

291 Patents
291II Patentability
291II(D) Anticipation
291k72 Identity of Invention
291k72(1) k. In General. Most Cited Cases
Patent anticipation requires showing that each limitation of claim is found in single reference, either expressly or inherently. 35 U.S.C.A. § 102.

[12] Patents 291 ⚿72(1)

291 Patents
291II Patentability
291II(D) Anticipation
291k72 Identity of Invention
291k72(1) k. In General. Most Cited Cases
Although disclosure of genus in prior art is not necessarily anticipating disclosure of every species that is member of that genus, very small genus can be anticipating disclosure of each species within genus. 35 U.S.C.A. § 102.

[13] Patents 291 ⚿66(1.24)

291 Patents
291II Patentability
291II(D) Anticipation
291k63 Prior Patents
291k66 Operation and Effect
291k66(1.24) k. Process, Method, and Apparatus Claims in General. Most Cited Cases
Prior art disclosure of method of synthesizing difluoromethane (CH_2F_2) within broad temperature range and ingredient ratios did not anticipate patent calling for synthesis within narrow subset of temperature range, partially overlapping ingredient ratio, and specific contact times; prior disclosure of large genus did not anticipate claimed species.

[14] Patents 291 ⚿97

291 Patents
291IV Applications and Proceedings Thereon
291k97 k. Patent Office and Proceedings Therein in General. Most Cited Cases
Patent may be rendered unenforceable for inequitable conduct if applicant, with intent to mislead or deceive examiner, fails to disclose material information or submits materially false information to Patent and Trademark Office during prosecution.

[15] Patents 291 ⚿97

291 Patents
291IV Applications and Proceedings Thereon
291k97 k. Patent Office and Proceedings Therein in General. Most Cited Cases
Party asserting patent's unenforceability due to inequitable conduct must prove threshold level of materiality and intent by clear and convincing evidence.

[16] Patents 291 ⚿97

291 Patents
291IV Applications and Proceedings Thereon
291k97 k. Patent Office and Proceedings Therein in General. Most Cited Cases
Patent applicant's intent to deceive examiner cannot be inferred solely from fact that information was not disclosed; there must be factual basis for finding of deceptive intent.

[17] Patents 291 ⚿97

291 Patents
291IV Applications and Proceedings Thereon
291k97 k. Patent Office and Proceedings Therein in General. Most Cited Cases
Failure of patent applicant to disclose full English translation of prior art Japanese patent was not inequitable conduct, such as would render patent for method of chemical synthesis unenforceable, absent showing of intent to deceive; applicant disclosed translated abstract of patent, and did not mischaracterize it when discussing prior art with examiner.

Patents 291 ⚿328(2)

291 Patents
291XIII Decisions on the Validity, Construction, and Infringement of Particular Patents
291k328 Patents Enumerated
291k328(2) k. Original Utility. Most Cited Cases
3,644,545. Cited as Prior Art.

Patents 291 ⚿328(2)

291 Patents
291XIII Decisions on the Validity, Construction, and Infringement of Particular Patents
291k328 Patents Enumerated
291k328(2) k. Original Utility. Most Cited Cases
5,900,514. Valid and Not Infringed.

***992** Thomas G. Rowan, Jones Day, of New York, New York, argued for plaintiff-appellant. With him on the brief were ***993**Daniel L. Malone, Eric C. Stops, and Gasper J. LaRosa.

Richard D. Harris, Greenberg Traurig LLP, of Chicago, Illinois, argued for defendant-appellee. With him on the brief was Brad R. Bertoglio.

Before LOURIE, RADER and DYK, Circuit Judges.

Opinion for the Court filed by Circuit Judge LOURIE.

Opinion concurring in part and dissenting in part filed by Circuit Judge DYK.

LOURIE, Circuit Judge.

Atofina appeals from the final decision of the United States District Court for the District of Delaware granting judgment in favor of Great Lakes Chemical Corporation ("Great Lakes") that Great Lakes did not literally infringe U.S. Patent 5,900,514 (the " '514 patent"); claims 1, 2, 6, 7, 9, and 10 of the '514 patent were anticipated by Japanese publication 51-82206 ("JP 51-82206"); and the '514 patent was unenforceable because of inequitable conduct. *Atofina v. Great Lakes Chem. Corp.*, Civ. No. 02-1350 (D.Del. March 23, 2005) ("*Final Judgment* "). We affirm the district court's claim construction of the term "chromium catalyst" and hence its determination of no literal infringement. However, because the district court clearly erred in finding that JP 51-82206 was an anticipatory reference meeting all the limitations of claims 1, 2, 6, 7, 9, and 10 of the '514 patent and also clearly erred in finding that the applicants of the patent intended to deceive the United States Patent and Trademark Office ("PTO"), we reverse its holdings of invalidity because of anticipation and unenforceability because of inequitable conduct.

BACKGROUND

The '514 patent is entitled "Synthesis of Difluoromethane," and was issued to Elf Atochem, which subsequently became Atofina, as assignee. The invention is directed to a method of synthesizing difluoromethane (CH_2F_2) through the gas phase fluorination of methylene chloride (CH_2Cl_2), with hydrogen fluoride (HF), in the presence of an amount of oxygen (O_2), within a particular temperature range, and with a chromium (Cr) catalyst. Claim 1 requires that the process be conducted in the presence of 0.1 to 5 moles of oxygen per 100 moles of methylene chloride, at a temperature of between 330 and 450 °C, with a "bulk or supported chromium catalyst." '514 patent, col. 7, ll. 20-25. The remaining asserted claims, 2, 5, 6, 7, 9, and 10, add further limitations: claim 2 further narrows the range of oxygen to methylene chloride ratios; claim 6 adds a requirement that the methylene chloride, oxygen, and hydrogen fluoride be in contact with the catalyst for a time between 0.01 and 10 seconds; claim 7 adds a pressure limitation requiring between 1 and 20 bars absolute; claim 9 is the same as claim 1 but with a different transition phase; and claim 10 is the same as claim 1 but with the addition of the contact time limitation from claim 6. *Id.*, col. 7, ll. 26-27, col. 8, ll. 3-11, 14-28.

In 1993, Great Lakes began manufacturing difluoromethane using a mixed metal catalyst consisting of a chromium compound with another element that the district court referred to as Agent X,[FN1] carried out in the presence of 1.1 to 1.2 moles of oxygen per 100 moles of methylene chloride,***994** at a temperature of 150 to 350 °C, and at a pressure between 5.5 and 7.6 bars absolute. *Atofina v. Great Lakes Chem. Corp.*, Civ. No. 02-1350, slip op. at 18 (D.Del. Feb. 23, 2005) ("*Opinion* "). The reactants in the process are in contact with the catalyst for approximately 10 seconds. *Id.* Agent X apparently enhances the selectivity of Great Lakes' fluorination reaction, as well as the catalyst life, but the process would not work in the absence of chromium. *Id.*, slip op. at 18, 20.

> FN1. Throughout this opinion, we will refer to a component of Great Lakes' proprietary catalyst formulation as Agent X.

On July 1, 2002, Atofina filed a complaint in the United States District Court for the District of Delaware accusing Great Lakes of infringing of the '514 patent. Great Lakes filed an answer and a counterclaim, alleging noninfringement, invalidity, and unenforceability because of inequitable conduct. After a bench trial, the court concluded that (1) Great Lakes did not infringe the '514 patent; (2) claims 1, 2, 6, 7, 9, and 10 were anticipated by JP 51-82206; (3) claim 5 of the '514 patent would not have been obvious in view of the prior art; (4) the '514 patent was not invalid for lack of enablement or failure to disclose the best mode; and (5) the '514 patent was unenforceable because of inequitable conduct. *Id.*, slip op. at 68. The court's holdings as to infringement, invalidity because of anticipation, and unenforceability because of inequitable conduct are at issue in this appeal.

First, the district court relied on the specification, the prosecution history, and dictionaries to construe the term "chromium catalyst" to mean "a substance that alters the velocity of a chemical reaction without itself being consumed, where the only catalytically active material is chromium without the addition of metal oxides, alkali metal fluorides, or non-inert additives." *Id.*, slip op. at 28-29. The court then determined that Great Lakes' catalyst did not meet the "bulk or supported chromium catalyst" limitation because it contained a non-chromium substance, Agent X, that was catalytically active or at the very least a non-inert additive that had been disclaimed in the specification. *Id.*, slip op. at 35-36. In addition, the court found that Great Lakes' catalyst contained a metal oxide other than chromium oxide that had been disclaimed by the applicants of the '514 patent during prosecution. *Id.*, slip op. at 36-37.

Second, the district court held that claims 1, 2, 6, 7, 9, and 10 were anticipated by JP 51-82206. *Id.*, slip op. at 39-45. Relying on *Titanium Metals Corporation v. Banner*, 778 F.2d 775, 782 (Fed.Cir.1985), the court determined that the broader temperature range of 100 to 500 °C recited in JP 51-82206 anticipated the narrower temperature range of 330 to 450 °C disclosed in the '514 patent. *Opinion*, slip op. at 41. The court also found that the additional limitation in claim 2, that the oxygen to methylene chloride molar ratio be between 0.5 percent and 3 percent, was anticipated by JP 51-82206's disclosure of part of that range (0.001 to 1 percent oxygen to methylene chloride molar ratio), again relying on *Titanium Metals*. *Id.*,

slip op. at 42. Furthermore, the court determined that although JP 51-82206 does not mention the contact times disclosed in claims 6 and 10, it nevertheless anticipates those claims because the "contact times may be calculated based on the information provided in the examples of JP 51-82206." *Id.*, slip op. at 43-44. The court also held that JP 51-82206 provides an enabling disclosure of the claimed process. *Id.*, slip op. at 45-47.

Finally, the district court held that the '514 patent was unenforceable because of inequitable conduct. *Id.*, slip op. at 58-67. The court first found that the fully translated version of JP 51-82206, which was not submitted to the PTO, was highly ***995** material "because it anticipate[d] all the limitations of claims 1, 2, 6, 7, 9, and 10 of the '514 patent." *Id.*, slip op. at 62, 66. The court then determined that Atofina intended to deceive the PTO based on its failure to disclose the full English translation of JP 51-82206, which it had in its possession. *Id.*, slip op. at 64. The court also based its finding of intent on Atofina's alleged misrepresentations to the PTO that JP 51-82206 disclosed a catalyst containing "chromium oxide and optionally other metal oxides" without mentioning that JP 51-82206 disclosed a catalyst of pure chromium oxide, as well as Atofina's alleged mischaracterizations of JP 51-82206 with respect to the scope of the reference and the contact times used in the reference. *Id.*, slip op. at 64-65. After balancing materiality and intent, the court concluded that the '514 patent was unenforceable because of inequitable conduct. *Id.*, slip op. at 67.

The district court entered judgment in favor of Great Lakes on March 23, 2005. Atofina timely appealed, and we have jurisdiction pursuant to 28 U.S.C. § 1295(a)(1).

DISCUSSION

[1][2][3][4] Claim construction is an issue of law, *Markman v. Westview Instruments, Inc.*, 52 F.3d 967, 970-71 (Fed.Cir.1995) (en banc), that we review *de novo*, *Cybor Corp. v. FAS Techs., Inc.*, 138 F.3d 1448, 1456 (Fed.Cir.1998) (en banc). The district court's determination of infringement, in contrast, is a question of fact that we review for clear error. *Centricut, LLC v. Esab Group, Inc.*, 390 F.3d 1361, 1367 (Fed.Cir.2004). Anticipation is also a question of fact that we review for clear error. *Hoover Group, Inc. v. Custom Metalcraft, Inc.*, 66 F.3d 299, 302 (Fed.Cir.1995). Finally, we review a district court's ultimate determination of inequitable conduct for abuse of discretion, and its threshold findings regarding materiality and intent to mislead for clear error. *Brasseler, U.S.A. I, L.P. v. Stryker Sales Corp.*, 267 F.3d 1370, 1379 (Fed.Cir.2001). "A finding is 'clearly erroneous' when although there is evidence to support it, the reviewing court on the entire evidence is left with the definite and firm conviction that a mistake has been committed." *United States v. U.S. Gypsum Co.*, 333 U.S. 364, 395, 68 S.Ct. 525, 92 L.Ed. 746 (1948).

I. *Infringement*

On appeal, Atofina argues that the district court incorrectly construed the term "chromium catalyst." Atofina asserts that the correct construction of "chromium catalyst" is a substance which causes the reaction to take place in which chromium is the catalytically active metal. Atofina also contends that the court erred in excluding all "metal oxides" and "non-inert additives" from the meaning of "chromium catalyst" based on statements in the specification and in the prosecution history. According to Atofina, the court read those statements out of context and they did not amount to a "clear and unmistakable" surrender of subject matter. In addition, Atofina contends that the court misread the Buckman reference, U.S. Patent 3,644,545, in excluding alkali metal fluorides. Atofina then argues that under its construction of "chromium catalyst," Great Lakes' process infringed the '514 patent because the process would not have worked without chromium. Atofina further asserts that the patent covers catalysts made from chromium derivatives, and that the catalyst Great Lakes uses is a chromium derivative.

Great Lakes responds that the district court correctly construed "chromium catalyst" to exclude metal oxides, non-inert additives, and alkali metal fluorides. According***996** to Great Lakes, Atofina disclaimed catalysts containing catalytically active substances other than chromium and catalysts containing non-inert additives. As support for its argument, Great Lakes points to statements in the specification that

the catalyst in the '514 patent is "based on pure chromium (without the addition of another metal oxide)" and Atofina's assertions in the prosecution history that it was "unnecessary to employ special additives" to increase the selectivity of its catalyst, and that the claims excluded "utilization of a combination catalyst, such as that taught by Tsuji," which disclosed a chromium-indium catalyst. Great Lakes then argues that it did not infringe the '514 patent because its catalyst contains Agent X, a catalytically active material other than chromium or alternatively a non-inert additive.

[5][6] We agree with Great Lakes that the court did not err in finding a lack of infringement. Our primary focus in determining the ordinary and customary meaning of a claim limitation is to consider the intrinsic evidence of record, *viz.*, the patent itself, including the claims, the specification and, if in evidence, the prosecution history, from the perspective of one of ordinary skill in the art. *Phillips v. AWH Corp.*, 415 F.3d 1303, 1312-17 (Fed.Cir.2005) (en banc). At the same time, *Phillips* confirmed that courts may " 'rely on dictionary definitions when construing claim terms' " and that "[d]ictionaries ... are often useful to assist in understanding the commonly understood meaning of words." *Id.* at 1322 (quoting *Vitronics Corp. v. Conceptronic, Inc.*, 90 F.3d 1576, 1584 n. 6 (Fed.Cir.1996)). We have also stated, however, that "the court must ensure that any reliance on dictionaries accords with the intrinsic evidence: the claims themselves, the specification, and the prosecution history.... [I]n those circumstances where reference to dictionaries is appropriate, the [court's] task is to scrutinize the intrinsic evidence in order to determine the most appropriate definition.' " *Free Motion Fitness, Inc. v. Cybex Int'l, Inc.*, 423 F.3d 1343, 1348-49 (Fed.Cir.2005) (citing *Phillips*, 415 F.3d at 1314, 1322-24).

As an initial matter, we conclude that the district court properly relied on scientific and technical dictionaries to construe the ordinary and customary meaning of the term "catalyst" as "a substance that alters the velocity of a chemical reaction without being consumed." Because there is no suggestion that the intrinsic evidence defines the term "catalyst," one may look to technical dictionaries for assistance in determining that term's meaning to a person of ordinary skill in the art. *Phillips*, 415 at 1318 ("Because dictionaries, and especially technical dictionaries, endeavor to collect the accepted meanings of terms used in various fields of science and technology, those resources have been properly recognized as among the many tools that can assist the court in determining the meaning of particular terminology to those of skill in the art of the invention. Such evidence, we have held, may be considered if the court deems it helpful in determining 'the true meaning of language used in the patent claims.' "). The McGraw-Hill Dictionary of Scientific and Technical Terms 307 (4th ed.1989) defines a catalyst as a "[s]ubstance that alters the velocity of a chemical reaction and may be recovered essentially unaltered in form and amount at the end of the reaction." The district court correctly accepted that ordinary and customary meaning.

[7] Having agreed with the court's definition of the term "catalyst," we also agree with its construction of "chromium catalyst" as a catalyst where the only catalytically active material is chromium without***997** the addition of metal oxides or non-inert additives. The intrinsic record supports that interpretation. The specification states that "it has now been found that there is a temperature range in which a catalyst based on *pure chromium (without the addition of another metal oxide)* can produce, in the presence of oxygen," difluoromethane. '514 patent, col. 2, ll. 54-59 (emphasis added). The specification further explains that "it is necessary to have a catalyst containing solely chromium." *Id.*, col. 7, ll. 7-12. In addition, the specification makes clear that it is "unnecessary to employ special additives to increase [the fluorination reaction's] selectivity; the elimination of additives employed in the mixed catalysts enables the manufacture of the catalyst to be simplified and thereby its cost to be reduced." *Id.*, col. 3, ll. 10-14. We note that the use of the term "chromium" in the patent includes chromium oxide as it refers to "solely chromium (catalysts A and B)," and catalyst A is chromium oxide. *Id.*, col. 7, ll. 8-9.

[8] The prosecution history confirms a construction of "chromium catalyst" that excludes metal oxides and non-inert additives. As discussed in *Phillips*, the meaning of the claim language may be limited by a disclaimer in the specification or prosecution history.

415 F.3d at 1316-17. Here, the applicants' statements in distinguishing their claimed "bulk or chromium catalyst" over prior art are a disclaimer of claim scope as to metal oxides and non-inert additives. The '514 claims were initially rejected on August 6, 1997, as obvious over the prior art. The applicants responded on December 30, 1997, that "the claims recited the phrase 'consisting essentially of' which would exclude the utilization of a combination catalyst, such as that taught by [the Tsuji reference, EP 629440]. Nowhere in the applicants' disclosure is there mentioned a combination of chromium and indium catalyst. Rather the applicants' disclosure in comparative examples 2 and 3 indicates *the criticality of utilizing chromium catalyst alone rather than in combination with other metal components.*" (emphasis added).

The applicants also pointed out that contrary to what might be expected from the prior art, its chromium-based catalyst carried out the fluorination reaction without a decrease in the selectivity of the reaction and that it was "therefore unnecessary to employ special additives to increase its selectivity." The applicants repeated these assertions in their August 20, 1998, response to the PTO's second rejection. In addition, the applicants stated that "pure chromium means without the addition of a metal oxide" and that "[c]ontrary to what might be expected from the prior art, the present invention makes it possible to employ a catalyst based solely on chromium to carry out this fluorination reaction." We thus agree with the district court that the applicants' statements are a disclaimer of claim scope as to catalysts containing metal oxides and non-inert additives. *Phillips,* 415 at 1317 ("[T]he prosecution history can often inform the meaning of the claim language by demonstrating how the inventor understood the invention and whether the inventor limited the invention in the course of prosecution, making the claim scope narrower than it would otherwise be.").

The district court also interpreted "chromium catalyst" as excluding catalysts containing alkali metal fluorides. The basis for the district court's construction as to "alkali metal fluorides" is the applicants' statements in the prosecution history that "[t]he recent claims exclude the utilization of an alkali metal fluoride noted in column 1, line 59 of Buckman, [U.S. Patent 3,644,545]." Buckman, which is entitled "Improved***998** Vapor Phase Fluorination Procedure in the Presence of Catalyst and Alkali Metal Fluoride," discloses a fluorination reaction "in the presence of a catalyst, when such reaction is carried out in the presence of an alkali metal fluoride." '545 patent, col. 1, ll. 54-59. While the Buckman specification apparently differentiates between the catalyst and the alkali metal fluoride in naming illustrative substances that may be employed as catalysts (Cr_2O_3, CrF_3, and AlF_3) versus alkali metal fluorides (KF and NaF) that should also be present in the reaction, but perhaps not as catalysts, and the applicants may thus have not intended to exclude an alkali metal fluoride as a catalyst, we are not prepared to find error in the district court's exclusion of alkali metal fluorides from its claim construction, as an alkali metal fluoride is clearly in the reference that the applicants were distinguishing. *Id.,* col. 2, ll. 47, 55.

[9] We reject Atofina's argument that the district court erred in its construction of "chromium catalyst" because the applicants' statements regarding "metal oxides" were intended to distinguish only nickel-chromium catalysts, not Agent X-chromium catalysts. That the applicants only needed to surrender nickel-chromium catalysts to avoid a prior art reference does not mean that its disclaimer was limited to that subject matter. "To the contrary, it frequently happens that patentees surrender more through amendment than may have been absolutely necessary to avoid particular prior art. In such cases, we have held the patentees to the scope of what they ultimately claim, and we have not allowed them to assert that claims should be interpreted as if they had surrendered only what they had to." *Norian Corp. v. Stryker Corp.,* 432 F.3d 1356, 1361-62 (Fed.Cir.2005) (citing *Fantasy Sports Props., Inc. v. Sportsline.com, Inc.,* 287 F.3d 1108, 1114-15 (Fed.Cir.2002)). Here, the patentee spoke expressly to the meaning of "chromium catalyst," both in the specification and in the prosecution history, noting that the catalyst was limited to "pure chromium (without the addition of another metal oxide)." We therefore agree with the district court that Atofina surrendered all catalysts containing non-chromium metal oxides.

[10] Having affirmed the district court's claim con-

struction of the term "chromium catalyst," we also affirm its judgment of noninfringement. First, the court found that "[b]oth the Johnson Matthey report and the Synetix report demonstrated that defendant's catalysts contained several metal oxides including Agent X oxide." *Opinion,* slip op. at 36-37. While we have some question from reading the record whether Great Lakes' catalyst actually contains an Agent X oxide, we nonetheless will defer to the district court's finding of fact on that issue. It is not clearly erroneous. Second, we discern no clear error in the court's alternative determination that Agent X, if not a catalyst, was a non-inert additive. *Id.,* slip op. at 35 n. 13. Thus, because the district court made no clear error in its finding that Great Lakes did not infringe Atofina's properly construed claims, we affirm its judgment of non-infringement.

II. *Anticipation*

On appeal, Atofina argues that JP 51-82206 does not anticipate any claim of the '514 patent because it does not disclose the manufacture of difluoromethane, recited in the preamble of claim 1; its disclosure of a broader temperature range does not anticipate the specific temperature range claimed in the '514 patent; its disclosure of an oxygen to methylene molar ratio of 0.001 to 1.0 is not a disclosure of the claimed range of 0.1 to 5.0 percent; ***999** and it does not disclose the contact times required in claims 6 and 10. According to Atofina, the court's reliance on *Titanium Metals* was misplaced because that case stands for the proposition that a species can anticipate a genus, not the reverse.

Great Lakes responds that JP 51-82206 anticipates claims 1, 2, 6, 7, 9, and 10 of the '514 patent because the claimed ranges are within the disclosure of ranges in the prior art. According to Great Lakes, JP 51-82206's disclosure of a preferred temperature range of 150 to 350 °C encompasses the temperature range disclosed in the '514 patent of 330 to 450 °C. Great Lakes also contends that JP 51-82206's disclosure of the oxygen to methylene chloride molar ratios of 0.001 percent to 1.0 percent encompasses the ratios claimed in the '514 patent of 0.1 percent to 5.0 percent. In addition, Great Lakes argues that even though JP 51-82206 does not disclose the contact times as required by claims 6 and 10 of the '514 patent, the contact times are "typically and easily determined through calculation, by a person of ordinary skill in the art."

[11][12][13] We agree with Atofina that the district court clearly erred in finding that JP 51-82206 anticipates the '514 patent. Anticipation requires a showing that each limitation of a claim is found in a single reference, either expressly or inherently. *Perricone v. Medicis Pharm. Corp.,* 432 F.3d 1368, 1369 (Fed.Cir.2005). However, each limitation of the '514 claims is not in JP 51-82206. It is well established that the disclosure of a genus in the prior art is not necessarily a disclosure of every species that is a member of that genus. *See, e.g., In re Baird,* 16 F.3d 380, 382 (Fed.Cir.1994). There may be many species encompassed within a genus that are not disclosed by a mere disclosure of the genus. On the other hand, a very small genus can be a disclosure of each species within the genus. *In re Petering,* 49 C.C.P.A. 993, 301 F.2d 676, 682 (1962); *see also Bristol-Myers Squibb Co. v. Ben Venue Labs., Inc.,* 246 F.3d 1368, 1380 (Fed.Cir.2001) ("[T]he disclosure of a small genus may anticipate the species of that genus even if the species are not themselves recited."). That is not the case here, however. A temperature range of over 100 degrees is not a small genus and the range of temperatures of JP 51-82206 does not disclose Atofina's temperature range.

To find anticipation here, the district court relied on our opinion in *Titanium Metals.* The court stated that "the '514 patent's claim limitation of 330 to 450 °C is entirely within JP 51-82206's temperature range of 100 and 500 °C. Consequently, this limitation of claim 1 is also disclosed by JP 51-82206." *Opinion,* slip op. at 41. However, *Titanium Metals* stands for the proposition that an earlier species reference anticipates a later genus claim, not that an earlier genus anticipates a narrower species. 778 F.2d at 782. Here, the prior art, JP 51-82250, discloses a temperature range of 100 to 500 °C which is broader than and fully encompasses the specific temperature range claimed in the '514 patent of 330 to 450 °C. Given the considerable difference between the claimed range and the range in the prior art, no reasonable fact finder could conclude that the prior art describes the claimed range with sufficient specificity to anticipate this limitation of the claim. Because the court's

determination that JP 51-82250 disclosed the temperature range in claims 1, 2, 6, 7, 9, and 10 of the '514 patent was grounded in its erroneous application of *Titanium Metals*, we must reverse its finding of anticipation based on the temperature range.

Further, we reject Great Lakes' argument that the district court's finding of anticipation was correct because JP 51-*1000 82206 discloses a preferred embodiment using a specific temperature range (a species) that anticipates the '514 patent's claim of a broader temperature range (a genus). JP 51-82206 discloses a preferred temperature range of 150 to 350 °C that slightly overlaps the temperature range claimed in the '514 patent. But that slightly overlapping range is not disclosed as such, *i.e.*, as a species of the claimed generic range of 330 to 450 °C. Moreover, the disclosure of a range of 150 to 350 °C does not constitute a specific disclosure of the endpoints of that range, *i.e.*, 150 °C and 350 °C, as Great Lakes asserts. The disclosure is only that of a range, not a specific temperature in that range, and the disclosure of a range is no more a disclosure of the end points of the range than it is of each of the intermediate points. Thus, JP 51-82206 does not disclose a specific embodiment of the claimed temperature range.

The district court also clearly erred in finding that the claimed oxygen to methylene chloride molar ratio of 0.1 to 5.0 percent was disclosed in JP 51-82206. JP 51-82206 discloses an oxygen to methylene chloride ratio of 0.001 to 1.0 percent that overlaps but does not fall within the range of ratios claimed in the '514 patent. Moreover, the disclosure of a 0.001 to 1.0 percent range in JP 51-82206 does not constitute a specific disclosure of 0.1 percent to 5.0 percent, as Great Lakes asserts. Once again, although there is a slight overlap, no reasonable fact finder could determine that this overlap describes the entire claimed range with sufficient specificity to anticipate this limitation of the claim. The ranges are different, not the same. Indeed, the lower end of the ratio in the reference differs by a factor of one hundred from what is claimed. In addition, the disclosure of a 0.001 to 1.0 percent range is not a disclosure of the end points of that range. Thus, there is no anticipation. Because JP 51-82206 does not expressly or inherently disclose the claimed range of ratios, JP 51-82206 does not anticipate claims 1, 2, 6, 7, 9, and 10 of the '514 patent.

Finally, the district court clearly erred in finding that JP 81-82206 inherently discloses the contact times found in claims 6 and 10 of the '514 patent. Claims 6 and 10 require that the "gas phase mixture of methylene chloride, anhydrous hydrogen fluoride and oxygen is in contact with the catalyst for a time between 0.01 and 10 seconds." '514 patent, col. 8, ll. 6-11. Those contact times are not expressly found in JP 51-82206. Nor has Great Lakes shown that the contact times are inherently disclosed in JP 51-82206. The calculations Great Lakes points to as inherently disclosing the contact times are based on the first and second *examples* in JP 51-82206, which state the diameters and lengths of the reaction tubes and the flow rates, but do not say anything about any contact times. Because anticipation by inherent disclosure is appropriate only when the reference discloses prior art that must necessarily include the unstated limitation, JP 51-82206 cannot inherently anticipate the claims of the '514 patent. *Transclean Corp. v. Bridgewood Servs., Inc.*, 290 F.3d 1364, 1373 (Fed.Cir.2002). We therefore conclude that the district court clearly erred in determining that the contact times in claims 6 and 10 are disclosed in JP 51-82206.

Because the district court clearly erred in finding that JP 51-82206 discloses the temperature range, the range of oxygen to methylene chloride molar ratios, and the contact times claimed in the '514 patent, we reverse the district court's finding of anticipation.

III. *Inequitable Conduct*

On appeal, Atofina argues that the district court abused its discretion in its holding*1001 of inequitable conduct because the full English translation of JP 51-82206 that was not submitted to the PTO was not highly material and there was no basis for inferring an intent to deceive the PTO. According to Atofina, the court's sole basis for finding that the English translation of JP 51-82206 was highly material was that the reference anticipated several claims of the '514 patent (its materiality for obviousness purposes was not decided). Thus, Atofina contends that because the court's determination as to anticipation was clearly erroneous, the finding of materiality is also

erroneous. As to intent, Atofina also contends that the court failed to consider evidence of good faith, such as Atofina's reference in the prosecution history to page numbers of the full translation of JP 51-82206. Atofina notes that the Derwent Abstract of JP 51-82206 was disclosed to the examiner and JP 51-82206 was cited in the patent specification.

Great Lakes responds that the district court's findings of a high level of materiality and intent were supported by clear and convincing evidence, and thus the court correctly held that the '514 patent was unenforceable. Great Lakes argues that the full English translation of JP 51-82206 was highly material because it was anticipatory and it teaches the use of a pure chromium catalyst. Great Lakes also points out that Atofina's application for a European counterpart to the '514 patent was rejected for lack of novelty over JP 51-82206, and issued only after Atofina amended its claims to include an additional limitation. According to Great Lakes, the prosecution of the European counterpart to the '514 patent is evidence of the materiality of the full English translation of JP 51-82206. In addition, Great Lakes argues that Atofina acted with intent to deceive the PTO in failing to disclose the full English translation of JP 51-82206, mischaracterizing prior art references, and also withholding information regarding poor results during the pilot phase testing of the '514 patent process. Great Lakes also contends that there was no evidence of good faith by Atofina in its failure to disclose the full English translation of JP 51-82206.

[14][15] We agree with Atofina that the district court abused its discretion in its conclusion that inequitable conduct occurred. "A patent may be rendered unenforceable for inequitable conduct if an applicant, with intent to mislead or deceive the examiner, fails to disclose material information or submits materially false information to the PTO during prosecution." *Digital Control, Inc. v. Charles Mach. Works*, 437 F.3d 1309, 1313 (Fed.Cir.2006). "The party asserting inequitable conduct must prove a threshold level of materiality and intent by clear and convincing evidence." *Id.* Further, "materiality does not presume intent, which is a separate and essential component of inequitable conduct." *GFI, Inc. v. Franklin Corp.*, 265 F.3d 1268, 1274 (Fed.Cir.2001) (quoting *Manville Sales Corp. v. Paramount Sys., Inc.*, 917 F.2d 544, 552 (Fed.Cir.1990)).

[16] The issue here is whether Great Lakes proved intent by clear and convincing evidence. The district court inferred intent from the applicants' failure to disclose the full English translation of JP 51-82206 and its alleged mischaracterizations of that reference. However, the applicants' failure to disclose the full English translation of JP 51-82206 is not in and of itself enough to infer intent, even if the full English translation went beyond the Derwent Abstract, which is far from clear. *See Semiconductor Energy Lab. Co. v. Samsung Elecs. Co.*, 204 F.3d 1368, 1378 (Fed.Cir.2000) ("The duty at issue in this case is the duty of candor, not the duty of translation."). "Intent to deceive can not ***1002** be inferred solely from the fact that information was not disclosed; there must be a factual basis for a finding of deceptive intent." *Hebert v. Lisle Corp.*, 99 F.3d 1109, 1116 (Fed.Cir.1996).

[17] The district court's finding of intent additionally rests on three statements made by the applicants to the PTO: (1) the applicants' statement that JP 51-82206 discloses a catalyst containing "chiefly chromium oxide and optionally other metal oxides"; (2) the applicants' statement that a "person skilled in the art, who is looking for a means of fluorinating *a specific H containing halocarbon* (CH_2Cl_2) with good selectivity is therefore not prompted to consider the teachings of ... JP 51-82206"; and (3) the applicants' statement that "[t]hese specific process conditions effect a contact time of 10 seconds or less .... The short contact time is not taught in the applied references. Contact time indicated in the references are substantially in excess of this." *Opinion*, slip op. at 64-65 (emphasis in original). The court determined that the first statement misrepresented to the PTO that JP 51-82206 failed to disclose a catalyst of pure chromium oxide, the second statement mischaracterized JP 51-82206 by failing to identify that methylene chloride was a preferred starting material in that reference, and the third statement mischaracterized JP 51-82206 by failing to mention the inherent "contact times" allegedly disclosed in JP 51-82206.

We conclude that the district court clearly erred in finding that these three statements were sufficient to prove intent. First, the applicants' statement that JP

51-82206 discloses a catalyst containing "chiefly chromium oxide and optionally other metal oxides" is consistent with the text of the full English translation of JP 51-82206, which asserts that its catalyst is "mainly comprising chromic oxide." And the Derwent Abstract that was disclosed to the PTO also states that the catalyst is "mainly comprising trivalent chromium oxide." "Mainly" and "chiefly" are words with similar meanings. Thus, there was no real difference between the disclosed Derwent Abstract and the undisclosed full English translation of JP 51-82206 in this context.

While the dissent states that "some parts of the majority opinion appear to hold there was no misdescription because JP 51-82206 does not disclose pure chromium oxide," that is incorrect. JP 51-82206 does disclose the use of a catalyst containing pure chromium oxide, JP 51-82206 at 4 ("Not only pure Cr_2O_3 but also chromium oxides mainly comprising Cr_2O_3 are usable in the present invention."), and we do not state otherwise. Moreover, characterizing a catalyst, as Atofina did, as containing "chiefly chromium oxide and optionally other metal oxides" is not inconsistent with it possibly being pure chromium oxide. Further, the dissent states that "chiefly chromium oxide" does not mean "chiefly Cr_2O_3" because "JP 51-82206 clearly used the term 'chromium oxides' to include oxides other than Cr_2O_3." While the language "chiefly chromium oxide" does encompass chromium oxides other than Cr_2O_3, it expressly discloses Cr_2O_3. Therefore, no reasonable fact finder could conclude that the applicants misrepresented to the PTO that JP 51-82206 failed to disclose a catalyst of pure chromium oxide. Because the applicants' statement was consistent with both the full English translation of JP 51-82206 and the Derwent Abstract, the district court clearly erred in finding that it supported an inference of intent.

Second, the applicants' statement that a "person skilled in the art, who is looking for a means of fluorinating a specific H containing halocarbon (CH_2Cl_2) with good ***1003** selectivity is therefore not prompted to consider the teachings of ... JP 51-82206" was not an omission of the fact that the preferred starting material in JP 51-82206 was methylene chloride. The seven starting materials disclosed in JP 51-82206, *including methylene chloride,* are disclosed in the first column of the '514 patent. '514 patent, col. 1, ll. 58-59. The applicants also stated to the examiner that JP 51-82206 "mentions, among the reactions, the fluorination of CCl_4, $CHCl_3$, CH_2Cl_2 [methylene chloride], CCl_3F, C_2Cl_6, C_2Cl_4, and $C_2H_3Cl_3$" and that the "preferred starting materials contemplated [in JP 51-82206] ... include perhalogenated molecules as well as H containing molecules," which include methylene chloride. Based on the record, we conclude that the district court clearly erred in finding that the applicants' statement was an attempt to hide the fact that methylene chloride was a preferred starting material in JP 51-82206.

Third, the applicants' statement that "[t]hese specific process conditions effect a contact time of 10 seconds or less ... The short contact time is not taught in the applied references. Contact time indicated in the references are substantially in excess of this" does not support an inference of an intent to deceive. As discussed previously, JP 51-82206 does not disclose any "contact times." Therefore, the district court clearly erred in finding that applicants' statement was misleading.

Thus, because the district court clearly erred in its findings of fact relating to an intent to deceive the PTO, we conclude that its holding of inequitable conduct based on those findings was an abuse of discretion. We therefore reverse that holding. Inasmuch as we reverse on intent, we do not discuss materiality.

We have considered the parties' remaining arguments and find them unpersuasive.

CONCLUSION

We affirm the district court's conclusion of no literal infringement. The district court, however, clearly erred in finding that JP 51-82206 was an anticipatory reference meeting all the limitations of claims 1, 2, 6, 7, 9, and 10 of the '514 patent and also clearly erred in finding that Atofina intended to deceive the PTO so as to support a determination of inequitable conduct. We therefore affirm the court's judgment of no literal infringement, and we reverse its holdings of invalidity because of anticipation and unenforceability for inequitable conduct.

AFFIRMED IN PART AND REVERSED IN PART.

DYK, Circuit Judge, concurring in part and dissenting in part.

I join the court's opinion insofar as it affirms the district court's finding of no literal infringement and reverses as to invalidity. I also agree that the district court's inequitable conduct determination cannot stand. In my view, that determination rests on an erroneous finding of materiality based on a finding that JP 51-82206 anticipated claims 1, 2, 6, 7, 9, and 10 of the '514 patent. I would vacate and remand this issue so that the district court can reconsider the inequitable conduct claim free of this error. In my view the majority errs in deciding to reverse without remand.

The majority does not address the issue of materiality, but reverses the district court's inequitable conduct determination on the ground that the district court's intent finding was clearly erroneous. I do not disagree with the majority's***1004** determination that the district court's second and third grounds for finding intent were clearly erroneous. However, a key question on the issue of intent was whether the applicants misdescribed JP 51-82206 by stating that JP 51-82206 disclosed a catalyst "containing chiefly chromium oxide and optionally other metal oxides" as opposed to describing JP 51-82206 as disclosing a pure chromium oxide catalyst. (If it disclosed pure chromium oxide it would be highly material to the prosecution of this patent, even though not anticipatory.) Some parts of the majority opinion appear to hold that there was no misdescription because JP 51-82206 does not disclose pure chromium oxide and the applicants accurately stated that it contained "chiefly chromium oxide and optionally other metal oxides."

The district court stated that "JP 51-82206 specifies that the chromium catalyst can either be 'pure' chromium oxide or mainly chromium oxide." *Atofina v. Great Lakes Chem. Corp.*, Civ. No. 02-1250, slip op. at 4 (D.Del. Feb. 23, 2005); *see also id.* at 42. The finding that JP 51-82206 discloses pure chromium oxide is supported by the text of JP 51-82206 which states:

> The Cr_2O_3 catalyst usable in the present invention can be prepared by various processes .... [Describing calcinations processes]. Cr_2O_3 prepared by calcining a chromium compound containing chromium hydroxide is particularly preferred. *Cr_2O_3 prepared by these processes is not pure but it contains a small amount of other chromium oxides* and has an atomic ratio of O/Cr of about 1.4 to 1.7. *Not only pure Cr_2O_3 but also chromium oxides mainly comprising Cr_2O_3 are usable in the present invention.* A metal oxide other than chromium oxide such as an alkaline earth metal oxide can be added to the catalyst as another constituent.

J.A. 3037 (emphasis added). While JP 51-82206 may not disclose the use of pure Cr_2O_3, it certainly discloses the use of pure "chromium oxides" without the presence of other metals, as is required by the '514 patent. [FN1]

> FN1. Atofina appears to argue that "chiefly chromium oxide"-which is how it described the catalyst disclosed in JP 51-82206-means "chiefly Cr_2O_3." This is misleading. Chromium oxide is "[a] compound of chromium and oxygen; chromium may be in the +2, +3, or +6 oxidation state." *McGraw-Hill Dictionary of Scientific and Technical Terms* 390 (6th ed.2003); *see also Van Nostrand's Scientific Encyclopedia* 787 (9th ed.2002) (describing "[t]he three oxides of chromium" as "CrO, Cr_2O_3, and CrO_3"). Further, JP 51-82206 clearly used the term "chromium oxides" to include oxides other than Cr_2O_3.

What a prior art reference discloses or teaches is a question of fact. *Novo Nordisk Pharm., Inc. v. Bio-Tech. Gen. Corp.*, 424 F.3d 1347, 1355 (Fed.Cir.2005); *Winner Int'l Royalty Corp. v. Wang*, 202 F.3d 1340, 1349 (Fed.Cir.2000). On this record, I see no basis for disturbing the district court's factual finding that JP 51-82206 disclosed "pure" chromium oxide.

The majority ultimately agrees that JP 51-82206 does disclose pure chromium oxide, but nonetheless concludes that the description in JP 51-82206 is not misleading. I cannot agree. The district court found as a factual matter that Atofina, which was in possession

of a full-length English translation of JP 51-82206, misrepresented this aspect of the Japanese patent to the PTO. In the '514 patent, and in representations to the PTO, Atofina described JP 51-82206 as claiming a catalyst "containing *chiefly chromium oxide* and optionally other metal oxides." J.A. 1129 at 1:48-49 (emphasis added). The district court determined that Atofina's use of the term "chiefly" was misleading in that it implied that the Japanese patent only disclosed***1005** a chromium catalyst which included other components, rather than disclosing a catalyst containing chromium oxides. *Atofina,* Civ. No. 02-1250, slip op. at 64. The majority rejects the district court's interpretation, stating that "characterizing a catalyst, as Atofina did, as containing 'chiefly chromium oxide and optionally other metal oxides' is not inconsistent with it *possibly* being pure chromium oxide." Maj. Op. at 1002 (emphasis added). The majority thus appears to hold that the description is not misleading because it is not a direct misstatement. I fail to see how the majority can rule as a matter of law that the district court could not find that Atofina's reference is misleading because of the implication that it conveys. *See Semiconductor Energy Lab. v. Samsung Elecs. Co.,* 204 F.3d 1368, 1377 (Fed.Cir.2000) (affirming a finding of inequitable conduct where the patentee submitted a one-page, partial translation of a foreign reference because the partial translation focused "on less material portions" and "left the examiner with the impression that the examiner did not need to conduct any further translation or investigation"). In context, the withholding of information that JP 51-82206 did *in fact* disclose pure chromium oxide could itself support a finding of intent.

Under these circumstances, it is possible that the district court could properly find intent in connection with this withholding of JP 51-82206. I would afford the district court the opportunity to reconsider its inequitable conduct determination.

C.A.Fed. (Del.),2006.
Atofina v. Great Lakes Chemical Corp.
441 F.3d 991, 78 U.S.P.Q.2d 1417

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EXHIBIT 6

SUPPLEMENTAL AMENDMENT TO MAY 4, 2009 AMENDMENT
FILED IN RESPONSE TO NOVEMBER 3, 2008 OFFICE ACTION

Submitted: October 7, 2009

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Applicants: Michael Wayne Graham et al.